2001

Modified capillaries in CEC separation of antibiotics and AFM imaging

Helen Tran
San Jose State University

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

MODIFIED CAPILLARIES IN CEC SEPARATION OF ANTIBIOTICS AND AFM IMAGING

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

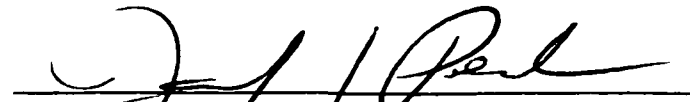
Master of Science

by

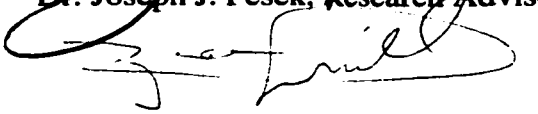
Helen Tran

December 2001

APPROVED FOR THE DEPARTMENT OF CHEMISTRY



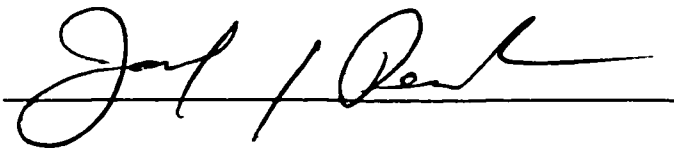
Dr. Joseph J. Pesek, Research Advisor



Dr. Roger Terrill, Committee Member

Dr. Elaine Collins, Committee Member

APPROVED FOR THE UNIVERSITY



ABSTRACT

MODIFIED CAPILLARIES IN CEC SEPARATION OF ANTIBIOTICS AND AFM IMAGING

By

Helen Tran

Modifying the inner-wall surface of a capillary in OTCEC is an approach to improve sample separation. The inner wall of a capillary is etched and bonded with either a C18 or a cholesteryl moiety or coated with a fluorosurfactant. In the separation of antibiotics, three comparisons of resolution are made: 1) between 20 μm and 50 μm capillaries, 2) between bare etched and etched chemically modified capillaries, 3) between bare etched and etched fluorosurfactant-coated capillaries. Resolution depends on the sample and pH. The best separations are achieved by the cholesteryl capillary for ampicillin/gentamycin and by bare etched capillaries for nystatin. The fluorosurfactant-coated capillary has improved resolution for gentamycin in comparison to the bare etched capillary. Comparable data are obtained on the 20 and 50 μm capillaries. Images of the chemically modified surfaces are obtained by AFM showing "pits" for the C-18 capillary and globular-shaped objects for the cholesterol capillary.

ACKNOWLEDGEMENTS

I would like to express my greatest appreciation toward my research advisors, Dr. Joseph J. Pesek and Dr. Maria Matyska Pesek, for introducing this research opportunity to me, for all of the valuable advices and guidance, and for being so patient and wonderful during my learning process. I wish to thank the National Science Foundation (Grant CHE 9625628) and the National Institutes of Health (Grant R15 GM 49452-01) for the support of this research.

I would also like to take this opportunity to thank Dr. Roger Terrill and Dr. Elaine Collins for being my committee members and for all the helpful suggestions. I want to thank Dr. Jane Frommer of International Business Machine (IBM) for granting me the privilege of using the AFM and for helping me with the scanning.

I would not have completed my study without the support of my family and friends. I want to thank my mom and my brothers for all the financial and mental support that I could ever receive. Many thanks should also be sent to a wonderful and special friend of mine, Clarke Chen, for all of his love, understanding, patience, and technical support.

Finally, a word of "thank you" is for my research group's friends: Ludmila, Huy, Haihong, LiHua, Sophie, Salma, Kelly, and others. I enjoyed the good times and also will never forget the hard times that we had gone through together. All your encouragements have always been the comfort and motivation that helped me to achieve better, and they will always be remembered in my heart.

TABLE OF CONTENTS

LIST OF FIGURES

Page

1. INTRODUCTION

1.1. Capillary Electrochromatography

Capillary Electrophoresis (CE) is a separation technique where samples are separated in a narrow-bore capillary (20 to 100 μm inner diameter) in the presence of an electric field. Since its introduction in the late 1960's (1), CE has slowly emerged to be an accepted and powerful separation method today. With the recent interest in the Human Genome Project, CE is also considered to be an applicable method for protein separation due to its high resolution, sensitivity, and small-scale sample requirement. For example, CE separation was reported in capillary electrochromatography (CEC) studies of transferrin and other protein mixtures (2). From some perspectives, CEC is a combination of both High Performance Liquid Chromatography (HPLC) and gel electrophoresis. The capillary serves as a separation medium where the sample and the stationary phase interact with each other similar to an HPLC column. In CEC, the sample movement is by the applied voltage as in gel electrophoresis whereas in HPLC, the sample elutes through the column at different rates depending on its interaction with the stationary phase in the column. However, unlike HPLC, the inside wall of a capillary in CEC can also be modified with either hydrophilic or hydrophobic compounds to improve the sample-stationary phase interaction as well as solvent flow, and in comparison to HPLC, CEC provides higher resolution. The separation of antibiotics is such an example where it has been found that HPLC results in a single sample peak while CEC results in two or three peaks for the same sample.

A few techniques complementary to CE are Capillary Zone Electrophoresis (CZE), Capillary Electrochromatography (CEC), Micellar Electrokinetic Capillary Chromatography (MECC), Affinity Capillary Electrophoresis (ACE), Capillary Isoelectric Focusing (CIEF), and Isotachophoresis (ITP). CEC is employed as the separation method in this study. However, it is important to discuss CE theory because it will assist in the fundamental understanding of the above mentioned techniques. A more detailed description will be provided for CE and CEC while the rest of the techniques will be discussed briefly. The basic concept of CE is based on sample separation by differential migration of charged species. The main components of a CE system include a capillary, buffer reservoirs, a UV detector, and power supply (Figure 1). Both ends of capillary are immersed in two identical buffer reservoirs. A detector is placed at some point between the two reservoirs. Solvent will move from the anode (inlet reservoir) to the cathode (outlet reservoir) through the capillary by electroosmosis. The distance of capillary from the inlet reservoir to the detector can be varied according to choice. This distance is critical since it is the length where samples will migrate and be separated before reaching the detector. Too short a capillary distance may not bring a good separation while too long a capillary distance may result in diffusion and long migration time. A window is made on the capillary by removing the polyimide outer coating of the capillary. The exposed part of the capillary is usually a small distance (~ three millimeters). When the voltage is applied, positively charged species in the solvent will move toward the cathode end. Some of these species may also interact with the negatively charged silica surface. It should be noted that the silica surface contains

silanol groups, which can be easily ionized as the buffer pH reaches 3 or above. This ionization at the wall forms a double layer with the inner layer referred as the Stern or Helmholtz layer. Next to this layer, the mobile outer layer, called the Gouy-Chapman layer, contains the positively charged species, which continuously move inside the capillary toward the anode creating a flow of solvent inside the capillary called the electroosmosic flow, or EOF (Figure 2). Solvent in a CE system has a "plug flow" in comparison to the "laminar flow" in High Performance Liquid Chromatography (HPLC) where solvent movement is the result of pressure, not EOF. The laminar flow in HPLC has a bow-shape or a parabola shape because the center of the mobile phase moves faster than the sides; whereas, the solvent-wall interaction slows down the movement at the sides. The plug flow, on the contrary, is driven by EOF. The charged buffer species in the capillary migrate at the same rate creating a vertically flat front similar to moving a plug inside the capillary. The EOF characteristics are one of the advantages of CE, which also include efficient heat dissipation, high applied- electric field, short analysis time, and low band diffusion. When the voltage is applied, the positively charged sample species move through the capillary at different migration rates depending on their total charge and size, which results in separation. The sample movement is called the electrophoretic flow. As a sample species passes by the window, its absorbance is recorded by the UV detector, which sends a signal to trigger further data analysis converting electronic signals to sample peaks as the output in the chromatogram. Samples exit the capillary to the outlet buffer reservoir.

CEC uses a CE system but with a modified capillary. CEC can utilize either

packed or open tubular capillaries. In the case of a packed capillary, porous silica beads with attached hydrophobic groups (e. g. C8, C18) are normally used to fill the capillary. In the case of open tubular CEC (OTCEC), the capillary wall is modified chemically to attach organic moieties such as liquid crystals (3, 4) or C18 (5); in some cases, cyclodextrin was also employed for chiral separation (6). The separation mechanism is based on the interaction between the solute and the bonded-stationary phase. Packing the capillary is a difficult process due to the small diameter of the inner bore. Air bubble formation is one major problem for packed CEC. OTCEC avoids the packing problems but it also has low EOF due to the masking of silanol groups by the coatings. The OTCEC approach in this study uses a different capillary modification process (Figure 3). It applies an etching step to the capillary prior to the modification. The etching process increases surface area and thus improves EOF. The capillary is etched with ammonium hydrogen difluoride at high temperature to create a rough surface (7). The modification includes two steps of silanization and hydrosilation (Figures 4 and 5). In the first step, the etched surface is modified to form a hydride monolayer by reaction with triethoxysilane (TES). This hydride surface reacts with the selected organic moiety to form the bonded phase in the second step. Some applications for a C18-modified capillary in CEC are the separation of tetracyclines (7) and N-nitrosodiethanolamine in cosmetics (8).

In addition to chemical modification of the capillary wall, coating the inner surface is another alternative. Coating, in this case, refers to lining a surface with the selected organic moiety. Coating the capillary wall is a simpler method for modification

rather than the above etching and chemical modification. It saves both time and effort. For OTCEC, the coating is applied to the etched surface, not to the bare capillary surface as in CE. Surfactants were originally used to improve the solubility of proteins and have been selected for their low UV absorbance. An advantage of fluorosurfactants is their high stability and binding-activity; they remain stable and active at very low concentration. Coating with surfactants prevents wall interaction (or wall adhesion) by protein samples (9) and/or enhances selectivity by providing a partition mechanism (10). Prevention of wall interaction is possible when cationic surfactants bind the silanol groups on the surface, or amphoteric surfactants form a bilayer at the capillary wall. The choice of surfactant is important since it determines the thickness of the adsorbed layer and hydrophobicity. The surfactant charge and structure are also critical because they affect the formation of the surface layer. Fluorosurfactants were selected in this study (Figure 6) for the study of the antiobiotics (Figure 7). Fluorosurfactants consist of both a hydrophillic and hydrophobic part. The hydrophobic part of the fluorosurfactant is lipophobic, unlike similar structures in hydrocarbon-type surfactants. Therefore, fluorosurfactants do not interact with oil and fat as in the case of hydrocarbon-type surfactants (11). Furthermore, cationic fluorosurfactants reverse the EOF. The positive head groups of cationic fluorosurfactants interact with the negative silanol groups on the capillary wall. This leaves the hydrophobic tails pointing outward from the wall to interact with the hydrophobic ends of other fluorosurfactants in the solvent. Once the bilayer is formed, the capillary wall carries the positive charges of the cationic fluorosurfactants head groups. Thus, this reverses the EOF (12). Mixtures of different

charged head groups (e.g. anionic surfactants, cationic surfactants, zwitterionic surfactants) in fluorosurfactants can also be used to shield the wall.

In comparison to CEC, CZE is the most common form of CE due to its simplicity. In CZE, the capillary is filled with a buffer and the sample is introduced as a zone at one end (1). No special capillary preparation is required, and additives or pH can be varied to control the selectivity. Proteins are usually run in CE at a pH above their pI to maintain a total negative charge to prevent wall interaction. The capillary surface can be given a net charge of zero by lowering the pH to acidic conditions for the separation of positively charged proteins. CZE has been reported to be a better separation technique for the tryptic-digested peptide Recombinant Human Insulin Growth Factor I (rhIGF-I) than HPLC (13). CZE was also used in studies of lysozyme, ribonuclease, cytochrome c3, and myoglobin (14).

The separation of neutral species commonly employs MECC. MECC is a CE system containing micelle-forming surfactants in the capillary. An anionic detergent like sodium dodecyl sulfate (SDS) is the most common additive to the running solvent. MECC separates neutral species by hydrophobic interaction. It is also applicable to the separation of charged species. Neutral species interact with micelles and are separated between the slow-moving micelles and the fast-moving buffer. Organic modifiers are also used together with the surfactant in MECC to improve separation.

Affinity Capillary Electrophoresis (ACE) provides a CE technique for studying macromolecule-ligand interactions. ACE separates samples based on the change in mobility when they interact with the affinity ligands. The advantage of ACE over the

traditional affinity method is that ACE requires no radio-labeling or a secondary reagent for quantitation. From the migration time of the complex and the ligand concentration, the equilibrium constant of the complex formation can be calculated. Capillary Isoelectric Focusing (CIEF) is similar to gel IEF. It mixes ampholytes and sample before introducing this mixture into the capillary. The ampholytes establish a pH gradient and the sample species are separated as they migrate according to their pI. The difference between CIEF and gel IEF is that the sample in CIEF must move pass the detection window.

1.2. Atomic Force Microscopy

Atomic Force Microscopy (AFM) is a surface detection technique, which was invented by Binnig, Quate, and Gerber in 1986 and subsequently commercialized in 1987. The principle of operation of AFM involves measuring atomic forces between a probe tip and the sample surface. In a simple explanation, when a tip is scanning over a sample surface, attraction and deflection from the surface causes changes from its original position. These changes are recorded and converted to topographic data. Figure 8 provides a more detailed description about how AFM works. As a tip approaches the sample surface, it is first drawn by van der waals attractive forces bending the tip toward the surface. As the tip and surface atoms get too close, their negatively charged electron clouds will repel each other pushing the tip away from surface. This phenomenon is called tip deflection. As the tip is deflected, it moves away from its original position. The new position of the tip is recorded and compared to the reference position. The

detector sends an error signal to the feed-back loop. The feed-back loop consists of an electronic system which controls the movement of the scanner. The scanner is made up of piezoelectric material; a ceramic composite that can expand or contract according to the applied voltage. The scanner can freely move in the x, y, or z directions. As the feedback loop receives the error signal, it generates a command to the scanner to reposition itself such that the detector can regain the original reference point. This action initiates the scanner movement either up, down. The reference signal, or set point, can be programmed in either a constant-force or a constant-height mode; constant-force is generally preferred. In the constant force mode, with the movement of the scanner, the feed-back loop will maintain a constant force value between the cantilever tip and the sample surface. A certain selected distance, between the tip and the sample surface, will be kept constant by the scanner if the constant-height mode is chosen. The motion of the scanner and the deflection of the cantilever, therefore, are nearly identical to a trace of the sample surface image. The computer software system records the movements of the scanner or the variation of the tip deflection and converts these measurements into topographic information. There are several types of tip-sample surface interactions in the contact mode of AFM including van der waals forces, capillary forces, and cantilever forces. Attractive and repulsive van der waals forces have been discussed. A capillary force occurs when there are water droplets or films, from moisture in the air or from condensed water, surrounding the tip or covering the sample surface. The tip will strongly interact with the water as it approaches the sample surface. It requires a much stronger force to pull the tip away from the surface during the scan if a capillary force is

involved. Capillary forces mask the true interaction force between tip and sample molecules, thus, resulting in inaccurate data. The force exerted by the tip onto the sample surface, or the cantilever force, depends on the cantilever deflection and spring constant. A vertical force is applied to the surface by the tip in the contact mode. Depending on the deflection of the tip, the scanner will respond by extension or contraction to deliver the appropriate forces to the surface.

Changes in the movement of the tip are detected by the beam-bounce detection system (Figure 9). A laser beam is reflected off a mirror to the cantilever tip. With the highly reflective surface of the cantilever tip, the laser beam is again reflected to the Position Sensitive Photodetector (PSPD). As the tip is deflected off the sample surface during the scanning process, it changes the reflective angle of the laser beam onto the PSPD. The detector records the change, makes a comparison to the reference, and sends an error signal to the feed-back loop, which monitors the movement of the scanner to keep either the selected force or height constant. The AFM technique can be performed in three modes: contact, non-contact, and intermittent (or also known as tapping) modes. In the contact mode, the tip is scanned very close to sample surface (a few angstroms away). In the non-contact mode, a larger distance of ten to a few hundred angstroms is set between the tip and the surface. The tapping mode scans the surface by an oscillating motion instead of a smooth zig-zag as in the case of the contact and non-contact modes. In the tapping mode, the cantilever vibrates creating a phenomenon where the tip moves toward the surface and then moves back out like the tapping motion of a finger on a table. The tapping mode has been reported to provide a better measurement for surface

roughness in comparison to the contact mode such as in the study of quartz and mica (15). The non-contact mode is applicable to soft materials because it will not damage the surface while the contact mode is more suitable for hard materials because of its close contact with the surface. The contact mode may cause irreversible damage or surface deformations. In addition, the contact mode can overcome capillary forcess better than the other modes.

The AFM tip plays a crucial part in the imaging process because it determines the interaction angle and, therefore, the resolution of the image. Tips are usually made of silicon or silicon nitride and are attached to the cantilever. Cantilevers are normally from 100 to 200 micrometers in length, 10 to 40 micrometers in width, and 0.3 to 3 micrometers in thickness (16). Tip shapes are also variable including triangular, pyramid, conical, etc. Different modes use different types of tips in order to achieve the optimum conditions. The non-contact or tapping mode requires a silicon nitride tip, which is more rigid than the silicon tip. The contact mode uses more flexible material like silicon because the tip is more easily deflected and will not deform the sample surface. The shape also contributes to analysis problems like tip artifact, also called tip imaging. This imaging occurs, for example as in the case of a triangular-shape tip and a 90 ° step-surface, when the end of the tip is not able to follow through the 90 ° angle of the step surface because the side of the triangular tip has contact with the edge of the step as the tip moves down the step. This contact of the side of tip prevents the end of tip to trace the 90 ° angle of the surface. The resulted image will show a slope, not a 90 ° angle-step. The true image, therefore, is not able to obtained due to tip imaging. AFM,

in general, is a complex technique due to the high number of instrumental variables.

This project focuses on the separation of antibiotics by various modified capillary surface in CEC. The antiobiotics tested are ampicillin, gentamycin, nystatin, and erythromycin. Different morphologies of the capillary surface had been achieved using the etching and silanization/hydrosilation methods. They include the etched, C18 (1-octadecene), cholesteryl (cholesteryl-10-undecanoate), and fluorosurfactant-coated capillary surfaces. A comparison between these modified capillaries for the separation of the antiobiotics has been carried out. In addition, a separation comparison between the 20-micron and 50-micron (i.d.) capillaries was also investigated. Furthermore, the inner-wall surface of the etched, C18, and cholesteryl-modified capillaries were examined by AFM. In a separate set of experiments, the modification process was also applied to the outer surface (or outer diameter) of several capillaries resulting in externally etched, C18-modified, and cholesteryl-modified surfaces. These externally modified capillary surfaces were also subjected to AFM examination.

2. EXPERIMENTAL

2.1. Apparatus

Capillary electrochromatograms were collected on two Perkin-Elmer/Applied Biosystems (Foster City, CA, USA) Model 270A-HT Capillary Electrophoresis systems, with a Version 5.10 operating system. The accompaning software included the HP Chem Station for LC and the HP Chem Station for CE270A ABI (Hewlett Packard, USA).

Atomic Force Microscopy data were obtained and analyzed by a Dimension 5000 (Digital Instruments) atomic force microscope by the tapping mode at International Business Machines Corporation (San Jose, CA, USA).

2.2. Capillary modification procedure

The etching/modification procedure has been discussed in the work of Pesek and Matyska as mentioned in the introduction (7). In the etching process, the capillary was cleaned by heating overnight with concentrated hydrochloric acid then washed with water, acetone, and diethyl ether. After drying with nitrogen for one hour, the capillary was filled with ammonium hydrogen difluoride and allowed to stand for 1 hour. The solvent for the ammonium hydrogen difluoride, methanol, was removed with nitrogen, and the capillary was heated in nitrogen at 300 °C for 2 hours and at 400 °C for one hour. It was then rinsed with methanol for one hour before drying for 30 minutes with nitrogen. Next, the capillary was preconditioned by passing a 6 mM ammonia solution for 20 hours and then it was flushed with 0.1 M HCl for 4 hours to remove any ammonia. The capillary was washed with deionized water for 2 hours and dried with nitrogen at 100 °C

for at least 20 hours. The silanization process was started by rinsing with dioxane, which was followed by treatment with a 1.0 M triethoxysilane (TES) solution for 90 minutes at 90 °C. The hydride surface was washed with THF for 2 hours and then dried with nitrogen. The capillary was washed with dry toluene. Hydrosilation was carried out by filling the capillary with 2 ml of pure 1-octadecene and 70 μl of hexachloroplatinic acid (Spiers catalyst), then heated to 60-70 °C for 1 hour. After heating, the solution was passed through the capillary for 45 hours at 100 °C. The capillary was then washed with toluene and THF for 1 hour and dried with nitrogen overnight at 100 °C. For external modification, the capillary was placed in an NMR tube with the appropriate solutions and subjected to same procedure

2.3. Coating of capillary with fluorosurfactants

The anionic (FSA) and amphoteric (FSK) fluorosurfactants were mixed into the CE running buffer at a concentration of 100 micrograms per milliliter using a Hamilton syringe. The mixture was filtered through a 0.45 μm filter disk. WARNING NOTE: FSA has a flash point of 21° C.

2.4. AFM sample preparation

The capillary was placed on a clean sheet of paper and fragmented into small pieces by crushing it with a metal rod (diameter of 1.9 cm). Under a dissecting microscope, fragments that had good exposure of the inner wall surface were selected by

a forcep and transferred onto a piece of two-sided tape, which was placed on the AFM metal disk sample holder. The whole sample disk was then placed on the AFM sample holder stand. For storage, these sample disks were placed in a closed cap box, which had a two-sided tape lining so the disks would not fall off or move around. For externally-modified surfaces, the capillaries were broken into approximately 1 centimeter-pieces and also placed in the sample holder disk as described. The AFM scan was carried out on the outside wall (also called outer-wall) of the externally modified capillary and on the inside bore (also called inner-wall) of the internally modified-capillary.

2.5. CEC sample preparation

Samples were prepared in buffer at a concentration of 1ug/mL. Ampicillin and erythromycin are supplied as white powders while nystatin is a very light yellow powder. One milligram of each material was dissolved in 1.00 milliliter of the appropriate buffer solution. Gentamycin was used at 10 mg/mL as provided by the manufacturer because further dilutions of the sample resulted in no detection in the sample-check runs. Nystatin and erythromycin samples were prepared at the same concentration but using 0.5 mL of buffer and 0.5 mL of 99% methanol. All prepared samples were filtered with a 0.2 μm filter disk before use.

Nystatin and erythromycin have low solubility in water. They formed suspensions but did not dissolve very well in aqueous buffers. In the comparison study of the 20 μm and 50 μm capillaries, nystatin and erythromycin were heated to 30 – 40 °C for a few minutes, sonicated for one minute, and suspended for approximately 16 hours in

buffer solution before being filtered through a 0.2 μm filter disk. In the other studies, nystatin and erythromycin were dissolved readily in methanol and buffer (50/50) and filtered with 0.2 μm filter disk.

2.6. Capillary Electrochromatography Procedure

CEC runs were carried out at 5, 10, 20, or 30 kilovolts (kV) and repeated at least twice for reproducibility. The applied voltage was increased from 20 kV to 30 kV when the ionic strength of the buffer was not sufficient to maintain a reliable EOF and decreased to achieve a better separation of peaks if they eluted too close to each other. Before and after each run, a one or two-minute flush was used, and the chromatogram was evaluated carefully for any sample peaks to ensure that there was a sufficient amount of sample and all components in the sample had gone through the capillary. This step, in which no voltage was applied, was used to determine detectibility (also known as a vacuum check). The injection time of the sample was varied from 0.1 to 5 seconds. Each run was carried out for 60 minutes, and only the part of the chromatogram, where sample peaks were present, was expanded for easier observation.

2.7. Materials

The internally modified silica capillaries have inner diameters of either 50 μm or 20 μm with the same outer diameter of 375 μm. The externally modified capillaries have outer diameters of 156 μm (bare and external etched) and 368um (external cholesteryl-

modified). Dr. Joseph Pesek 's research group (San Jose State University, Chemistry Department, CA, USA) modified these capillaries, which were supplied by Polymicro Technologies (Phoenix, AZ, USA). The antibiotics are ampicillin, gentamycin, nystatin, and erythromycin; all were obtained from Sigma (St. Louis, MO, USA). Four buffer solutions were used: 1) 30 mM phosphate/19 mM Tris, pH 2.14; 2) 375 mM 4-aminobutanoic acid (or GABA)/300 mM acetic acid, pH 4.41; 3) 19 mM Tris/30 mM potassium monophosphate, pH 7.40; 4) 100 mM Tris/150 mM boric acid, pH 8.14. Dilutions of 1:20, 1:10, and 1:5 were used as running buffers from the stock solutions. De-ionized water was obtained from a Millipore filtration system. The prepared buffers were filtered with either 0.2 μm or 0.45 μm filter disks before use. Fluorosurfactants Zonyl® FSA and Zonyl® FSK were obtained from Dupont Performance Chemicals (Wilmington, DE, USA).

3. RESULTS AND DISCUSSION

3.1. 20 μm vs. 50 μm capillary

Typical capillary electrochromatography uses fused silica capillaries of 50 μm to 100 μm (internal diameter). Smaller i.d. capillaries, in theory, should give better resolution due to more efficient solute-bonded phase interactions. This part of the study makes a direct comparison between 20 μm and 50 μm i.d. capillaries through the separation of four antibiotics at four different pH and buffer conditions. Both capillaries were etched and C18-modified.

The separation of ampicillin at pH 2.14 and 4.41 does show a slight but difference in resolution between the 20 μm and 50 μm capillaries (Figure 10). Two major peaks are found in all chromatograms. The 20 μm capillary data show better resolution at pH 2.14. The separation time between the two peaks in the 20 μm capillary chromatogram (pH 2.14) is 30 minutes compared to about 13 minutes in the 50 μm capillary (Figure 10C). For the separation at pH 4.41, the separation in the 50 μm capillary is approximately 10 minutes in comparison to 5 minutes in the case of the 20 μm capillary. This change in resolution in both pH values of the two capillaries may be due to a change in the EOF. As the pH increases, the EOF also increases. The sample peaks will elute faster resulting in the shorter separation time. Two very small peaks of about 1 milli- absorbance unit (mAU) are found in the 50 μm capillary chromatogram as well. These peaks might be from the ampicillin sample, which were later observed on the cholesteryl-modified

capillary. The chromatographic data at pH 7.40 and 8.14 for the 20 μm and 50 μm capillaries are not comparable due to low sample solubility (less than 1mAU). As shown in the chromatograms of the 20um capillary (Figure 11A), the baseline is very noisy, and the small peaks have an absorbance of about 0.2 mAU. This run was carried out for 60 minutes, and only these small peaks were observed. Another possibility is that these peaks are the result of an unstable baseline, or noise. The 20 μm capillary (pH 7.40) shows three clear peaks (Figure 11B) in comparison to the small, poorly defined peaks on the 50 μm capillary (Figure 11D). A sample check (or flushing the capillary with buffer) after the run on the 50 μm capillary showed that ampicillin sample was still in the capillary even after seventy minutes. A small amount of the sample did get through (the very small group peaks of about one milli-absorbance unit or less) while the majority of ampicillin was still retained. The 20 μm capillary shows three clear peaks (4 to 9 milli-absorbance units) with the same sample injection volume of 1 second.

In the separation of gentamycin, the chromatographic data cannot be compared directly between 20 μm and 50 μm capillaries due to the absence of the sample in the 20 μm capillary. No sample peak was observed in the sample check (Figure 12A). This absence of sample may be due to low sample solubility or adhesion of the sample to the capillary wall; more details will be discussed later. Gentamycin at pH 4.41 (20 μm capillary) shows a very small sample peak (~ 1mAU), which is still not significant for comparison purposes (Figure 12B). No sample peak was present in the vacuum check at pH 7.40 and 8.14 for gentamycin. A vacuum check refers to flushing the sample through the capillary using a vacuum (either 20 inches or 5 inches) with no applied voltage. This

is a quick way to check for the presence of the sample. If there is sufficient sample present, a peak will appear within about 3 minutes. All the samples were subjected to a vacuum check before running with voltage to ensure their presence. The 50 μm capillary, on the other hand, has a strong sample absorbance (Figure 12C, 12D and 13C, 13D). The 50 μm capillary gentamycin chromatograms show two to four significant sample peaks with absorbances ranging from approximately 20 to 80 mAU.

A similar observation of no sample detection was obtained for nystatin and erythromycin in both 20 μm and 50 μm-capillaries. The 20 μm capillary data showed no peaks in runs where a voltage was applied (Parts A and B in Figures 14 to 17). A likely possibility for the absence of the sample is due to their insolubility in buffer. Both nystatin and erythromycin appear insoluble in buffer when they were prepared at the concentration of 1mg/mL. They dissolve well in 100% methanol and partially dissolved in the 1:1 mixture of buffer and methanol. Nystatin was observed to dissolve partially in a methanol/buffer solution. Some small particles still remained undissolved. Erythromycin seemed to dissolve better than nystatin in the mixture. The 50 μm capillary data for nystatin show poorly resolved peaks blending into the instrumental noise and an unstable background (Figures 14C, 14D and 15C, 15D). The methanol/buffer solution improves the solubility of sample but does not significantly improve detection for the sample. For example, the sample check for erythromycin showed a very small to no sample peak (Figures 16A, 16B and 17A, 17B). There is no good explanation at this point why nystatin and erythromycin could not be detected even though they dissolved partially in the methanol/buffer solution. Furthermore, this

instrument was observed to display noise peak problems when the venting door was opened for temperature control. A noise peak resulted whenever the venting door was opened during the run. However, depending on the room temperature, it usually took one or two hours of operation before the instrument temperature surpassed the set temperature, which caused the venting door to open. Therefore, noise-peaks were observed in parts of some chromatograms but not in all. An unstable background may also be due to temperature fluctuations during the run.

The reason for the absence of the sample in the 20 μm capillary is not clear. The capillary was checked for the possibility of clogging and window misalignment by observing the flush output and running with a known sample. The capillary did not appear to be clogged since the inside-bore was clear under visual observation with a microscope, and liquid did go through the capillary upon manual flushing. A misalignment of the detection window was also ruled out because a known sample was successfully detected. Solute instability may be ruled out because the same sample was used in both capillaries (20 μm and 50 μm). Two possible reasons can be used to explain the detection failure in the 20 μm capillary. The narrow inner bore capillary size means that smaller amounts of sample will pass through the detection window in comparison to the 50 μm capillary. If the sample volume is too small, the detector may not be sensitive enough to detect the solute. Second, there is also a chance that the sample can be irreversibly adsorbed onto the inner wall surface. A small capillary i. d. such as 20 μm should provide better sample-bonded phase interactions than a larger i. d. capillary. In summary, the comparison between 20 and 50 μm capillaries was not completely

successful, but it did provide some clear separations like ampicillin at pH 8.14 on a 20 μm capillary. In general, the 50 μm capillary seems to result in better sample detection than the 20 μm capillary.

3.2. Etched, C18, and cholesteryl-modified capillaries

A comparison of the separation of antibiotics was made between three different inner-wall modified capillaries. The capillaries are the etched, C18-modified, and cholesteryl-modified capillaries. The etched capillary was the product of etching the inner-wall surface at high temperature with ammonium hydrogen difluoride; no further modification with organic groups followed. Another etched capillary was modified by bonding with octadecene (C18) and is referred to as either the C18-modified or etched C18 capillary. The cholesteryl-modified capillary was produced by bonding cholesteryl-10-undecanoate to the etched surface; this modified capillary is also referred to as the etched cholesteryl capillary. The cholesteryl-modified capillary shows the best separation of ampicillin at pH 2.14 in comparison to the etched and C18 capillaries (Figures 18A, 18B, 18C). As mentioned before, HPLC analyses result in only one ampicillin peak under various experimental conditions. However, the present data for the cholesteryl-modified capillary in CEC shows 4 peaks at approximately 5, 9, 23, and 24 minutes. The electrochromatogram on the etched capillary also has 4 major peaks, but the last two are not resolved as well as in the cholesteryl-modified capillary. There are 6 small peaks (~ 1mAU) in both the electrochromatograms of the etched and cholesteryl-modified capillaries. Three of these small peaks are between the second and third major peak. The

other two or three small peaks are found between the first and second major peaks. These peaks could be real, and they have a very similar pattern in both chromatograms. The C18-modified capillary displays only two peaks in comparison to the other two capillaries. The cholesteryl-modified capillary has the longest retention time, and the etched capillary has the shortest retention time. This may be due to the fact that cholesteryl-modified capillary has a longer hydrophobic arm so it can interact more efficiently with the sample molecules than the C18 and etched capillaries. The separation of ampicillin at pH 4.41 shows two broad peaks at 15 and 19 minutes in the electrochromatogram on the cholesteryl-modified capillary (Figure 19). At low pH, the carboxyl group of the ampicillin is more likely to be protonated so ampicillin has a higher degree of hydrophobicity than at pH 4.41. The ampcillin sample, therefore, has poorer separation at pH 4.41 than at pH 2.14 by the cholesteryl-modified capillary. In fact, only two major peaks are found in the electrochromatograms of all three capillaries instead of the 4 peaks observed for these capillaries at pH 2.14. Both the etched and the C18-modified capillaries also show two very small peaks. They are at 3 – 4 minutes for the etched capillary and 5 – 7 minutes for the C18-modified capillary. This may be due to the structural difference of the bonded moieties. C18 has a shorter chain length than cholesterol so its selectivity for hydrophobic molecules is not as effective. Ampicillin at pH 4.41, with lower hydrophobicity, is not retained as long as on the cholesteryl-modified surface because the sample is less hydrophobic at this pH so it does not have a stronge interaction with the most hydrophobic surface like the cholesteryl-modified surface (note that retention time in C18 is 20 –30 minutes and 15 –20 minutes in

cholesteryl). A similar explanation is applied to the etched capillary since the etched surface has fewer protonated silanol groups at high pH leading to lower hydrophobic interaction with the sample. The etched capillary has two major peaks and two minor peaks at pH 4.41 in comparison to four major peaks at pH 2.14. The separation of ampicillin at pH 7.40 shows one single broad peak for the C18 and cholesteryl-modified capillaries; the etched capillary has two peaks (Figure 20). Poorer separation at higher pH for the C18 and cholesteryl-modified capillaries probably is due to less interaction with ampicillin since it is more deprotonated. There is a short retention time, ranging from 2 to 6 minutes for both the C18 and cholesteryl capillaries. An unstable baseline and a noise peak (at 0.7 minute) are also observed in the etched capillary. Peaks are poorly resolved at pH 4.41 for all capillaries (a single peak instead of four peaks for the etched and C18 capillaries and two peaks for the cholesteryl-modified capillary). At high pH, pH 8.14, silanol groups are completely ionized leading to a higher EOF. The ionized silanol groups interact better with the positively charged species on the buffer creating a strong double layer, which moves the solvent faster to the cathode. The retention time for ampicillin, for all capillary conditions, at pH 7.40 and 8.14 is much shorter than at lower pH (Figures 21A, 21B, 21C). The cholesteryl-modified capillary displays three broad peaks at pH 8.14 in contrast to the one broad or two broad peaks at lower pH. There is no clear explanation at this point for the reason why the cholesteryl-modified capillary shows a better separation of ampicillin at high pH.

A proposed hypothesis for the better separation of ampicillin at pH 2.14 involves the molecular structure at this low pH. At pH 2.14, the carboxyl group of

ampicillin is protonated. This protonation increases the hydrophobicity of ampicillin resulting in better interaction with the nonpolar groups on the modified surfaces of the capillary. Better separtion, therefore, is obtained. On the etched surface, the silanol groups are also protonated at pH 2.14 forming a less polar medium as in the modified capillary. Very small sample peaks are observed in the case of the C18 capillary. The electrochromatograms have the same pattern of three peaks, but an unstable baseline and noise peaks imply some instrumental problem at this point.

Another example of good resolution by the cholesteryl-modified capillary is shown in the separation of gentamycin at pH 2.14 (Figure 22). The separation on the cholesteryl-modified capillary results in five peaks in comparison to four peaks and one peak for the C18 and the etched capillaries, respectively. The C18-modified capillary shows clear separation of 4 peaks but is not as well resolved as on the cholesteryl-modified capillary. At pH 4.41, the C18 capillary seems to give a slightly better resolution than the cholesteryl-modified capillary; there are five or six peaks in comparison to four peaks. However, these five peaks are not very well resolved on the etched capillary (Figure 23). The resolution on the etched capillary improves to five peaks instead of the one peak observed at pH 2.14, but they are not well separated. As the pH increases, the resolution improves for the etched and C18-modified capillaries while it gets worse for the cholesteryl-modified capillary. Three to four peaks are found on both the etched and the C18-modified capillaries while only one broad peak is obtained on the cholesteryl-modified capillary. The data for the cholesteryl-modified capillary also contains instrumental noise peaks and a noisy baseline.

The separation of nystatin on the etched capillary, at all four pH values was significantly better than on other modified capillaries. At pH 2.14, the chromatogram on the etched capillary shows three peaks while on the cholesteryl-modified capillary there is one broad peak, and the C18 capillary has a chromatogram with an unstable baseline (Figure 26). At pH 4.41, the etched capillary has four very well -resolved peaks while the other two capillaries contain multiple unresolved peaks and peak groups (Figure 27). At other pH values, the etched capillary provides one single peak in comparison to two or three peaks by C18 and cholesteryl-modified capillaries. However, the detection with C18 and cholesteryl-modified capillaries is problematic. Some chromatograms show an unstable baseline, a noisy baseline, or noise - peaks (Figure 26B, 27C, 28C). These problems may be due to temperature fluctuations during the run, door noise, or other instrumental noise. A commonly observed negative dip is also found in most of the electrochromatograms of nystatin. This negative dip probably is the solvent peak because methanol is included in the buffer. The etched capillary seems to provide the best resolution at pH 4.41. Out of the four pH values, 4.41 is where all three modified capillaries have the best separation; the etched capillary shows four clear peaks while both the C18 and cholesteryl-modified capillaries show multiple peaks with a similar peak-pattern. At other pH values, the separation is either poor or non-existant. Even at a lower pH of 2.14, the nystatin electrochromatogram only shows three peaks with poor resolution. However, it is not clear at this point why nystatin has an optimal separation on pH of 4.41 and the etched capillary.

The separation of erythromycin was not successful probably due to low

concentration and instrumental problems. The etched and C18-modified capillaries show an unstable background (Figure 30A and 30B), and the chromatogram at pH 2.14 shows one small peak on the cholesteryl-modified capillary but with a very noisy baseline (Figure 30C). This rise in the baseline may be due to temperature fluctuations, which occurred during the run, or solvent effects from injecting the sample. The chromatograms at pH 4.41 show no peak in the sample-check (or vacuum check) for the etched and cholesteryl-modified capillaries (Figure 31A and 31C). The negative dip is due to the sample solvent, methanol, as it passes the detector. The result implies that the sample was injected into the capillary, but it might not be soluble enough to show any absorbance. Unstable baselines and the absence of sample are observed at pH 7.40 and 8.14 for the etched and cholesteryl-modified capillaries (Figure 32A, 32C, 33A, and 33C). The C18-modified capillary shows a very small peak at pH 7.40, but this is also uncertain because the chromatogram is full of instrumental noise peaks (Figure 32B). More instrumental noise is observed at other pH values for the C18-modified capillary (Figure 33C).

In summary, in the comparison between the 20 μm and 50 μm capillaries, the 20 μm capillary has detection problems with respect to the 50 μm capillary. The possible reasons are low sensitivity in detection or the sample adsorption on the capillary wall. Due to the small diameter of the 20 μm capillary, the amount of sample was below the detection limit. Furthermore, such a small amount of sample could totally absorb to the capillary wall. The 50 μm capillary, therefore, results in better sample detection in comparison to the 20 μm capillary. However, ampicillin is observed to have the best

separation on the 20 μm capillary at pH 8.14 due to good solubility of ampicillin in the buffer providing sufficient sample for detection in all cases. Gentamycin is best separated by the 50 μm capillary at pH 2.14. Erythromycin is probably separated better by a 50 μm capillary at pH 7.40 and nystatin also on the 50 μm capillary at pH 4.41.

As for the modified capillaries, the cholesteryl capillary at pH 2.14 results in the best separation for ampicillin and gentamycin. Nystatin is best separated is on the etched capillary whereas erythromycin was not successfully analyzed due to low solubility.

These antibiotic separations using various modified capillaries serve as an overview for the analysis on different types of columns in OTCEC. It reveals some optimal separation conditions for a few samples in terms of pH and type of capillary.

3.3. Etched and etched-fluorosurfactant coated capillaries

It has been reported that surfactants bind silanol groups on a capillary surface more efficiently than most common basic compounds (17). Anionic and amphoteric surfactants were used in this study to determine their effect in CEC separations. Fluorosurfactants FSA and FSK are found to be soluble at pH 4.41 and are not soluble at pH 7.40 or 8.14. The solubility at pH 2.14 requires further investigation. After transferring the mixture of fluorosurfactant and buffer at pH 2.14 into a beaker, a thin, oil-like layer was observed on the bottom of the container. This result indicates that an FSA layer was formed on the bottom of the beaker and did not dissolve.

The separations were carried out with a 50 μm-etched capillary at pH 4.41. The

data between the etched-fluorosurfactant coated capillary and the etched capillary were compared. For ampicillin, there is no significant improvement in resolution when comparing the etched to the etched-FSA-coated or etched-FSK-coated capillary (Figures 34A, 34B and 34C). Ampicillin has two major peaks and two minor peaks in the chromatogram. Four peaks are observed in all three chromatograms. The two observed differences between the etched and etched-fluorosurfactant-coated capillaries are that the sample peaks in the etched-fluorosurfactant-coated capillaries were eluted later (e.g. six minutes for the first peak in the etched-fluorosurfactant-coated capillary in comparison to 3.5 minutes in the etched capillary) and the separation time between the two major peaks in the etched-fluorosurfactant-coated capillaries is also greater than in the etched-capillary (about 5 minutes apart for the etched-fluorosurfactant-coated capillaries and 1 minute for the etched-capillary). The fluorosurfactants can interact with the Gouy-Chapman layer at the capillary wall to form a bilayer. The anionic or amphoteric surfactants interact with the charged groups on the capillary wall providing a more efficient shield for the wall. Sample interaction, therefore, is improved leading to a longer retention time.

The etched-FSA-coated capillary provides significantly better resolution for the separation of gentamycin in comparison to both the etched and the etched-FSK-coated capillaries. Six peaks are resolved on the FSA capillary compared to five peaks (Figures 35A, 35B and 35C) on the other two capillaries. It is obvious that the resolution on the etched-FSA-coated capillary is better than the etched-FSK-coated capillary even though they both cover the capillary wall in a similar manner. This improvement in separation

may be due to a weak interaction between the hydrogen of the silanol groups and the negatively charged head groups (the carboxyl groups) of the FSA at pH 4.41, which leaves the hydrophobic ends extending out from the wall surface. These non-polar tails may have reduced the adsorption of the analytes to the capillary wall resulting in a better resolution; the protonated form of silanol may not be in high population at pH 4.41, but due to the acidic pH, some small percentage may still exist. In the case of the amphoteric FSK, its positively charged head groups are more likely to interact with the deprotonated silanol groups resulting in the negatively charge ends pointing away from the surface. These ends may shield the silica surface but may not be helpful in the separation. For the separation of nystatin, both etched-fluorosurfactant-coated capillaries do not result in an improved resolution (Figures 36A, 36B, and 36C). They show multiple peaks that are poorly resolved. The etched capillary still provides the best resolution of four clear peaks in comparison to the unresolved-multiple peaks on the fluorosurfactant-coated capillaries. The separation of erythromycin was not attempted due to low sample solubility in the buffer.

3.4. AFM inner-bore surface images of C18 and cholesteryl-modified capillaries

In this part of the study, images of the inner-bore surface of C18 and cholesteryl-modified capillaries (i.d. 50 μm and o.d. 375 μm) were taken by AFM. An optical view of the scanning process is shown in Figure 37. The length of most fragments is one millimeter (mm) or less. The inner-bore (50 μm i.d.) surface of the capillary fragment is

exposed. The light reflects off the surface forming a white line at the bore center. The very dark area that surrounds the bore is the body of the capillary. Some dark spots are observed on the inner-bore surface in this fragment. A separate scan on the outside of the bore, the main body of the capillary, also showed the presence of some dark spots. It was concluded that the dark spots are contaminants that are present on the bore surface. They are not characteristic of the bore surface itself. Even though the fragments were stored in a closed container, it is almost impossible to have a dust-free environment. Tiny glass fragments that were formed during the breaking of the capillary could also be attracted to the bore surface. Contamination, therefore, is highly likely because such tiny capillary fragments are not easily subjected to air or liquid -cleaning due to their small size and difficulty in handling. In Figure 38A, the sharp-edged white objects on the 5 μm-scan are most likely smaller capillary fragments that come from the breaking process due to the straight edges.

The C18-modified capillary inner-bore surface contains small, irregular-shaped areas, which are deeper than the surface because they appear darker in the image (Figure 38B). This image of the C18-modified surface agrees with the image of the same type of surface that was reported in the work of Pullen (18). A closer look at the 1 μm-scan (Figure 38C) shows the C18-modified surface looks more like a sponge. It consists of round, irregular shaped materials, which do not fully cover the surface. There are also uncovered areas which appear as holes or "pits" on the surface.

The apprearance of the cholesteryl-modified capillary inner-bore surface is much different than the C18 capillary. The 5 μm scan (Figure 39A) image reveals globular-

shaped objects. These objects group together to form masses (Figure 39B) that result in a complex structure on the modified surface. It is also shown that there are dark areas in the image. These "holes" may mean that the surface is not completely covered. The cholesteryl-modified inner-bore is more bulky than the C18-modified inner-bore surface. This difference in structure is probably due to the fact that cholesteryl 10-undecanoate is a larger and bulkier molecule than 1-octadecene. Holes and pits are observed in both cases. This may also mean that the surface is not covered 100% by the organic moiety in the modification process.

3.5. AFM outer-surface images of bare, etched, and cholesteryl-modified capillary

The biggest challenge with scanning the inner bore is gaining good access to the site. The two side- walls of a capillary fragment are most likely to be higher than the inner-bore surface. These high side- walls become the largest problem in getting the triangular-shape tip to contact the lower inner-bore surface. The cantilever arm can touch the side- wall surface and not be able to move further down while the tip has not yet reached the inner-bore surface.

As a complementary study to the internal modification of the capillary, external modification of the outer surface was carried out for comparison and for the advantage of scanning. The outer surface of the capillary was subjected to the same modification process. Figure 40 shows the optical image of the scanning process when the cantilever is on top of the whole capillary. A picture was taken when the microscope was coming

into focus with the top surface of the bare capillary (o. d. 156 μm; see Figure 41). The faint image of the inner-bore can also be observed. The focus was on top of the bore in this case; focusing on the top of capillary was necessary to locate the outer surface of the capillary for the tip to be placed properly. When the focus was on the bottom of the bore, striations were observed on the outer surface of the bare capillary (Figure 42). It is not clear whether this is due to a capillary characteristic from the production process or a lighting effect is responsible for this observation. Surface modification is observed on the outer surface of the etched capillary (Figure 43). The optical view of the etched outer surface shows a wavy line structure uniformly covering the whole surface while this structure is not observed in the bare (unetched) surface (Figure 44). A closer look at the etched outer surface by AFM is shown in Figure 45. The AFM images of the etched surface are composed of long, thick tubular features that blend together (Figure 45A). A 3D image of the etched surface is shown in Figure 46. The surface looks like as if it is composed of a compressed closed-packed structure. These results are consistent with the expected rough surface due to the etching process. A closer look at the 1 and 5 μm-scan images reveals some similarities between the etched outer surface and the cholesteryl-modified inner-bore surface. They both have large, globular features with holes on the surface. AFM images of the bare capillary (o. d. 156 μm) are full of round-shape objects that vary in size and are scattered on the surface. These features may be contaminants from tiny broken pieces of glass. Kaczmarek also did observe these round white objects scattered along a bare capillary surface studied by SEM (19). The cholesteryl-modified outer surface (o. d. 368um) is smoother than the bare and etched surfaces (Figure 47). It

is smooth with few protrusions (Figure 48).

A direct comparison between the bare, etched, and cholesteryl-modified capillary outer surface is shown in Figures 49 and 50 for 5 μm and 1μm-scan, respectively. By comparing the images, the surface roughness is in the order etched to bare to cholesteryl-modified from the highest to lowest degree of roughness. Because of such a large difference in the feature sizes, objects on the outer surface of C18 and cholesteryl-modified capillaries could barely be seen in comparison to the etched surface (Figure 49). Small objects on the bare surface are either a surface characteristic or contaminants. The appearance of the etched outer surface seems to be a true characteristic of the surface because of the uniformity and the expected roughness due from etching. The addition of cholesteryl groups onto this rough surface results in a smoother surface as observed in the modified capillary outer surface. This new topography probably comes from the cholesteryl groups filling in the "holes" on the etched outer surface resulting in smoother features. Figure 50, the 1 μm-scan, gives a more detailed look at the three surfaces. The small objects, on the bare and cholesteryl-modified capillary surfaces, have a round shape, and the enlarged portion of the etched capillary surface also seems to consist of many round-shaped objects, which cluster together and stack on each other to form the condensed close packed apperance.

4. CONCLUSIONS

In the comparison between 20 μm and 50 μm capillaries, the 50 μm capillary seems to have better sample detectability due to its larger diameter. The 20 μm capillary has a narrower inner diameter reducing the amount of sample seen by the detector. Thus, only a few of the samples are detected in the 20 μm capillaries (only ampicillin at pH 2.14 and 8.14 and gentamycin at pH 4.41). Wall adsorption of the sample may also be another factor that is responsible for sample loss in the 20 μm capillary. The unsuccessful separations by the 20 μm capillary does not mean its separation efficiency is less than that of the 50 μm capillary. The 20 μm capillary has been used successfully in CEC separation of lysozymes and cytochrome c as well as other biomolecules like tryptamine and serotonin as mentioned in the introduction. The 50 μm capillary results in the separation of antibiotics in most cases. Nystatin and erythromycin have low solubility under the experimental conditions used and, therefore, could not be easily detected. The second part of the comparison investigates different modification conditions of the capillary including the etched capillary, the C18-modified capillary, the cholesteryl-modified capillary, and fluorosurfactant-coated capillaries. In the comparison between the etched, C18-, and cholesteryl-modified capillaries, ampicillin is best separated at pH 2.14 by the cholesteryl-modified column. The separation of gentamycin is also best performed by the cholesteryl-modified capillary at pH 2.14. Nystatin has the best resolution by the etched capillary at pH 4.41. Erythromycin could not be detected very well probably due to low sample concentration. A particular separation depends on the optimal conditions due to the nature of the sample, the capillary, and other separation

conditions such as pH, temperature, and other instrumental factors. As described above, ampicillin was best detected at pH 2.14, gentamycin at 2.14, and nystatin at 4.41 by two different types of capillaries, etched and cholesteryl-modified capillaries. These successful separations at different pH values and capillary conditions imply that each antiobitic achieves an optimal interaction with a certain capillary surface at a particular pH. Ampicillin probably has the most efficient interaction in its protonated form with the non-polar, modified capillary surface at pH 2.14 leading to the best separation at this pH. However, there is no clear explanation for the good separation of gentamycin at low pH and for nystatin by the etched capillary. This study serves as a brief overview for several different types of capillary. It is difficult to create an optimal separation condition for all the antibiotics because each antibiotic is different in structure, size, and total charge; therefore, it is not possible to achieve a single experimental condition that will be suitable for all sample differences. This study reveals some optimal conditions for some samples in terms of pH and capillary-choice. All samples were subjected to the same conditions of buffer composition and pH value; therefore, the optimal separation conditions for each sample may require further investigation.

Fluorosurfactant FSA improves the resolution for the separation of gentamycin at pH 4.41. The fluorosurfactant FSA-coated capillary shows no significant change in resolution for other antibiotics. The fluorosurfactant FSK-coated capillary shows no improvement in resolution for any antibiotic in this study.

The AFM data show that the C18-modified capillary inner-bore has a surface, which is full of "pits". The cholesteryl-modified capillary inner-bore surface has

protruding globular objects. The outer surface is mostly smooth with some round objects for the bare capillary, a rough surface with protruding globular, tubular objects (like the inner-bore of the cholesteryl-modified surface) for the etched capillary, and a smooth surface with only a few small protrusions for the cholesteryl-modified capillary. The rough outer surface of the etched capillary is due to chemical etching, and the smoother outer surface of the cholesteryl-modified capillary is the result of the etched surface being filled with the organic moiety.

5. FUTURE WORK

This study has utilized anionic and amphoteric fluorosurfactants. It would be interesting to continue this study with a cationic fluorosurfactant or different combinations of fluorosurfactants. Many studies involving protein separations have used mixtures of cationic, anionic, and zwitterionic surfactants (20, 21). This study is only a brief overview of antibiotic separation using different modified capillaries. The optimal separation condition for each sample has only been generally established. More specific optimal conditions for each sample will require further investigations. A new buffer or method is probably necessary to improve the solubility of nystatin and erythromycin for better detection and a more valid comparison. The results of AFM provide very valuable information about the modified surfaces. A quantitative method should also be explored to evaluate the degree of modification.

REFERENCES

1. Cunico, R. L.; Gooding, K. M.; Wehr, T. *Basic HPLC and CE of Biomolecules.* Richmond: Bay Bioanalytical Laboratory Inc., **1998**. 259-300.

2. Pesek, J. J.; Matyska, M. T.; Swedberg, S.; Udiva, S. *Electrophoresis* **1999**, *20*, 2343-2348.

3. Pesek, J. J.; Matyska, M. T.; Muley, S. *Chromatographia* **2000**, *52*(7,8), 439-444.

4. Matyska, M. T.; Pesek, J. J.; Katrekar, A. *Analytical Chemistry* **1999**, *71*(24), 5508-5514.

5. Pesek, J. J.; Matyska, M. T.; Mauskar, L. *Journal of Chromatography A* **1997**, *763*, 307-314.

6. Pesek, J. J.; Matyska, M. T.; Menezes, S. *Journal of Chromatography A* **1999**, *853*, 151-158.

7. Pesek, J. J.; Matyska, M. T. *Journal of Chromatography A* **1996**, *736*, 255-264.

8. Matyska, M. T.; Pesek, J. J.; Yang, L. *Journal of Chromatography A* **2000**, *887*, 497-503.

9. Emmer, A.; Roeraade, J. *Journal of Liquid Chromatography* **1994**, *17*(18), 3831-3846.

10. Rodriquez, I.; Li, S. F. Y. *Analytica Chimica Acta* **1999**, *383*, 1-26.

11. Emmer, A.; Roeraade, J.; *Electrophoresis* **2001**, *22*, 660-665.

12. Emmer, A.; Jansson, M.; Roeraade, J. *Journal of Chromatography* **1991**, *547*, 544-550.

13. Rudnick, S. E.; Hilser Jr., V. J.; Worosila, G. D. *Journal of Chromatography A* **1994**, 672, 219-229.

14. Pesek, J. J.; Matyska, M. T.; Cho, S. *Journal of Chromatography A* **1999**, *845*, 237-246.

15. Simpson, G. J.; Sedin, D. L.; Rowlen, K. L. *Langmuir* **1999**, *15*, 1429-1434.

16. Howland, R.; Benetar, L. *A Practical Guide to Scanning Probe Microscopy 1993-1997.* Park Scientific Instruments.

17. Ye, M.; Zou, H.; Liu, Z.; Ni, J.; Zhang, Y. *Analytical Chemistry* **2000**, *72*(3), 616-621.

18. Pullen, P. E.; Pesek, J. J.; Matyska, M. T. *Analytical Chem.* **2000**, *72*, 2751-2757.

19. Kaczmarek, Mira. *Thesis, San Jose State University. Scanning Electron Microscopy Study of Etched Capillary Surfaces.* **1996**. 51.

20. Emmer, A.; Jansson, M.; Roeraade, J. *Journal of Chromatography A* **1994**, *672*, 231-236.

21. Hult, E. L.; Emmer, A.; Roeraade, J. *Journal of Chromatography A* **1997**, *757*, 255-262.

22. Chu, C.; Jonsson, E.; Auvinen, M.; Pesek, J. J.; Sandoval, J. E. *Analytical Chemistry* **1993**, *65*, 808.

23. Pesek, J. J.; Matyska, M. T.; Williamsen, E. J.; Evanchic, M.; Hazari, V.; Konjuh, K.; Takhar, S.; Tranchina, R. *Journal of Chromatography A* **1997**, *786*, 219-228.

24. Sandoval, J. E.; Pesek, J. J. *Analytical Chemistry* **1991**, *63*, 2634.

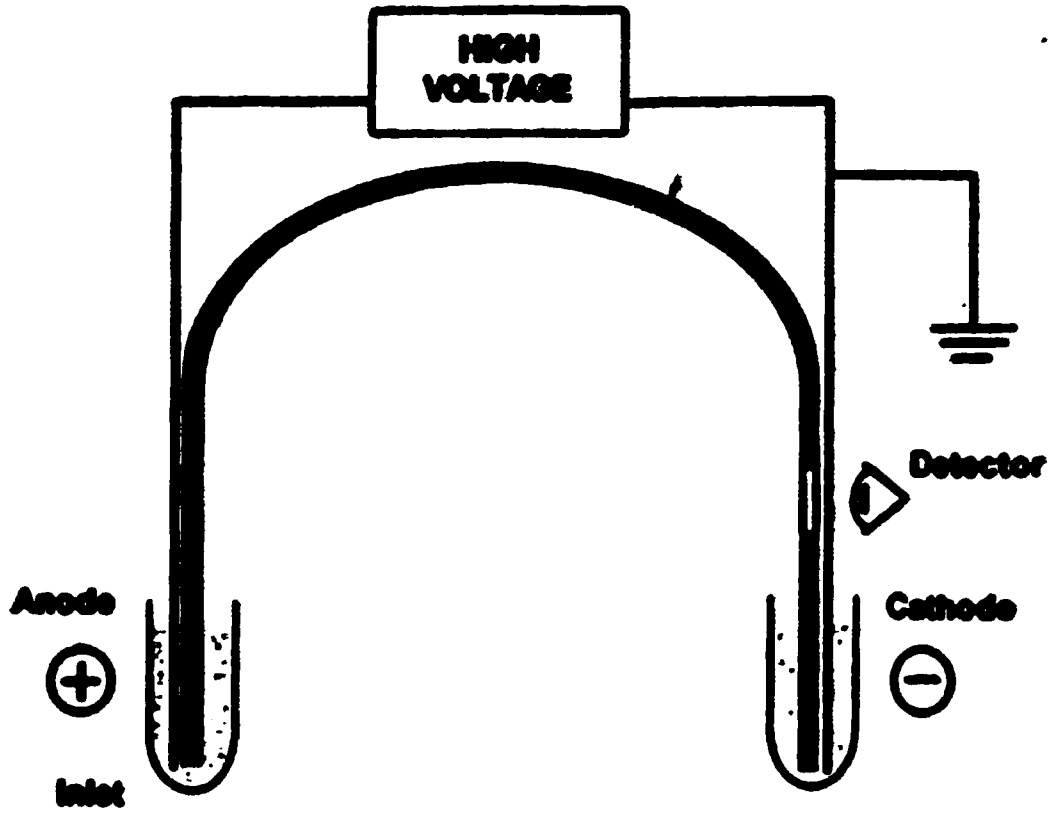


Figure 1 - Capillary Electrophoresis system (1).

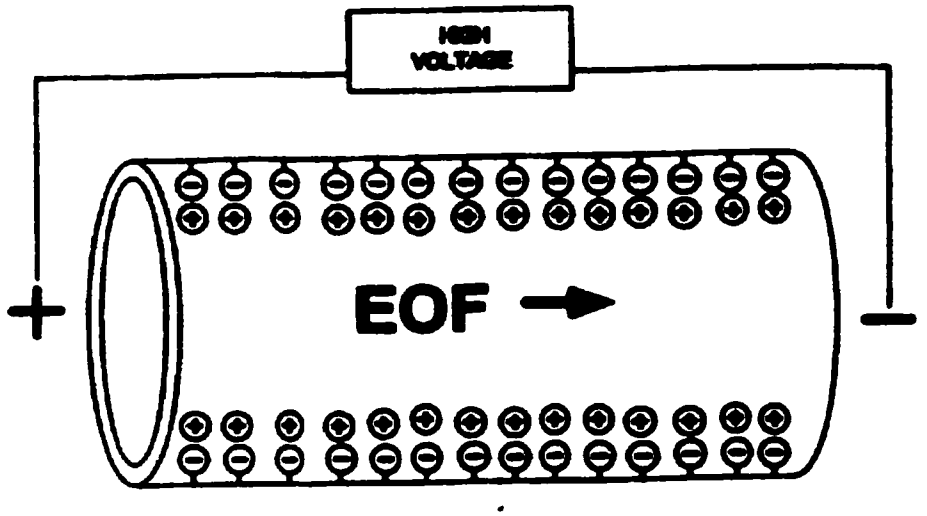


Figure 2 - Electroosmotic Flow direction of the Capillary Electrophoresis system (1).

ETCHING AND MODIFICATION PROCESS OF CAPILLARY INNER WALL SURFACE

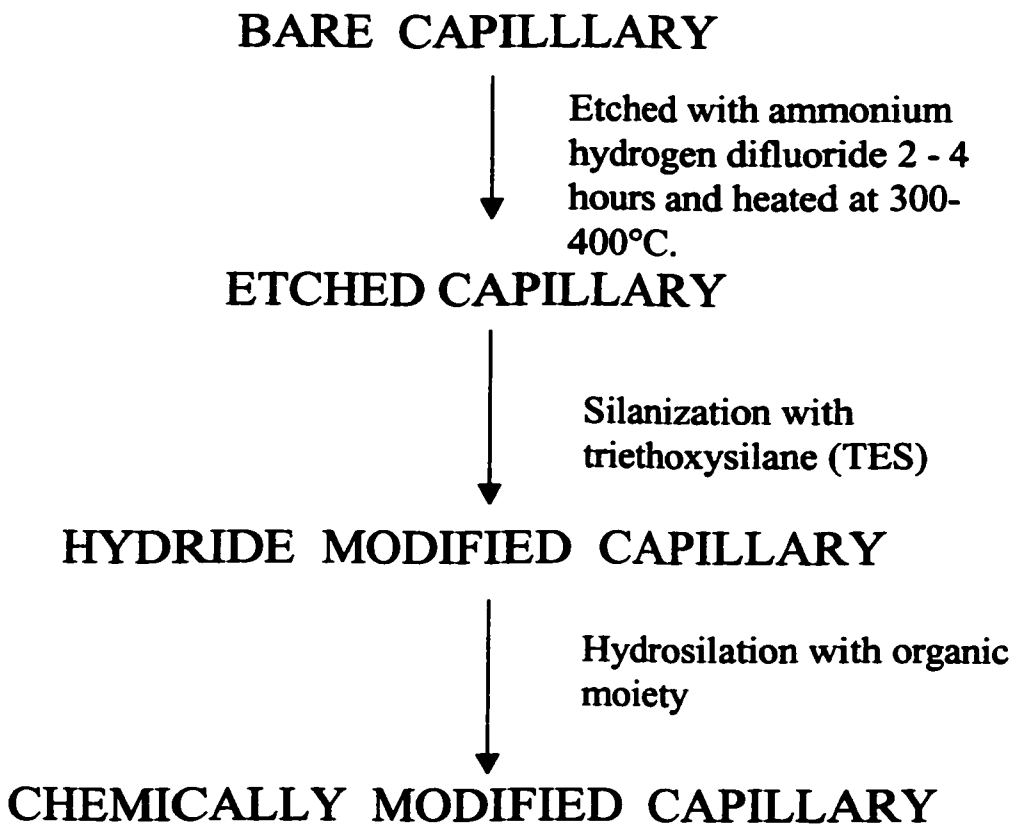


Figure 3 - General scheme of capillary modification process

CEC Capillary Derivatization

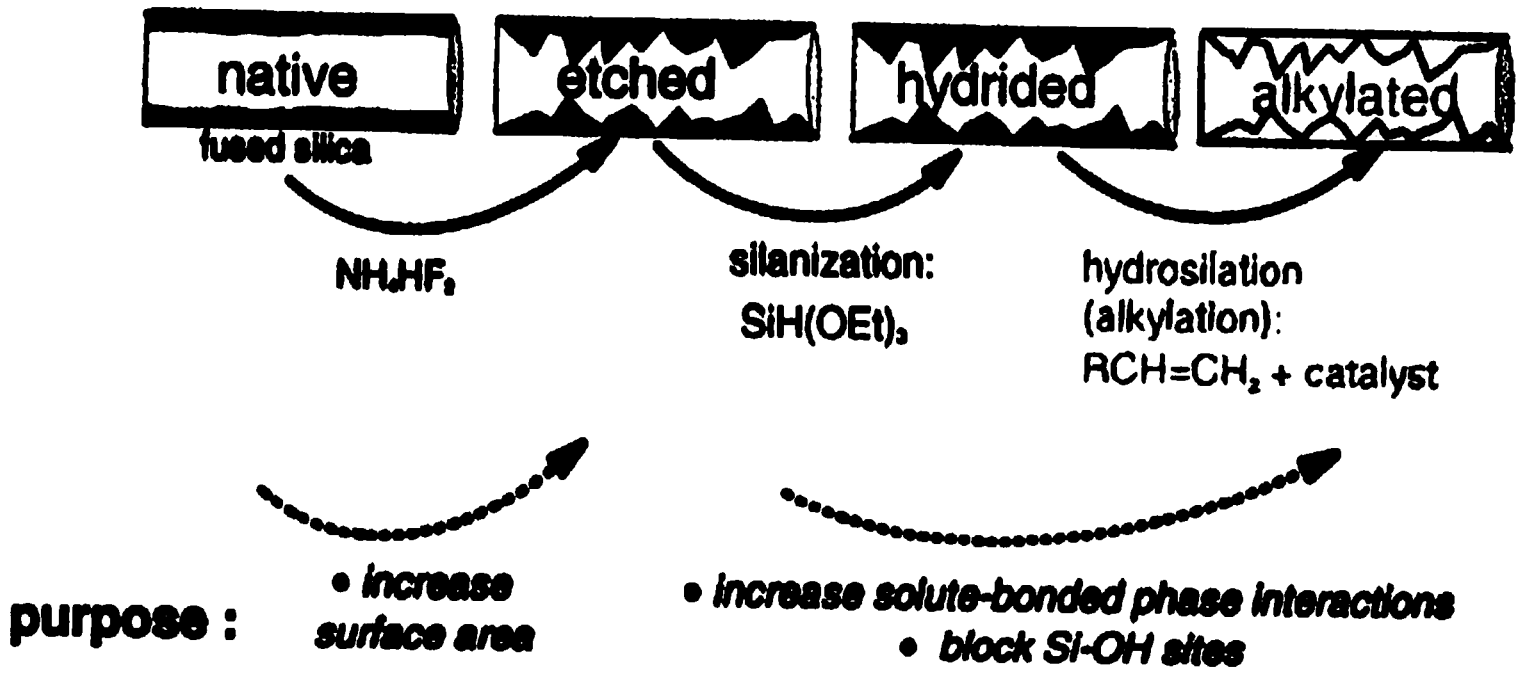
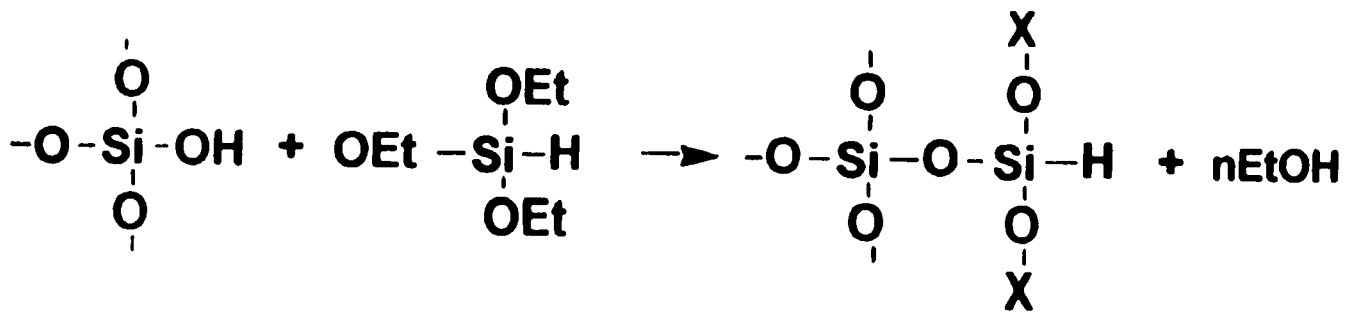
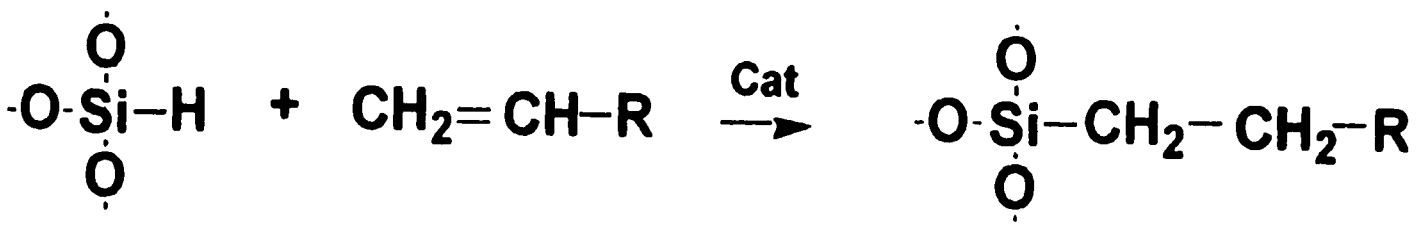


Figure 4 – Sequence of steps in capillary modification for CEC (printed through the courtesy of Pesek and Matyska, San Jose State University).

Silanization / Hydrosilation



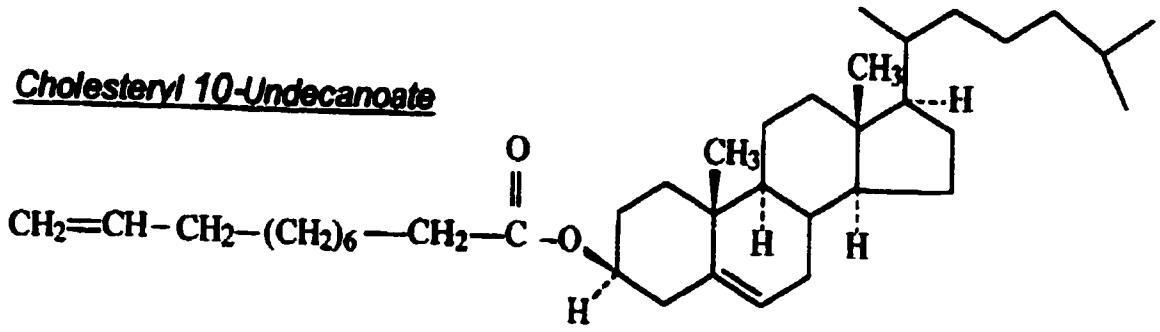
where X = Si or H and n = 1-3



Catalysts = Hexachloroplatinic acid (Pt) or AIBN - Azo-bis (isobutyronitrile) - free radical initiator

Figure 5 - Silanization and hydrosilation process (22, 23, 24)

Cholesteryl 10-Undecanoate



Octadecene

$$CH_2 = CH - CH_2 - (CH_2)_{14} - CH_3$$

Fluorosurfactant FSA

$$Rf\text{-}CH_2\text{-}\ CH_2\text{-}S\text{-}\ CH_2\text{-}CH_2\text{-}COO^-\ Li^+$$

where $Rf = F(CF_2CF_2)x$
$x = 1$ to 9

Fluorosurfactant FSK

$$Rf\text{-}CH_2\text{-}\ CHO(Ac)\text{-}CH_2\text{-}N^+(CH_3)_2\text{-}CH_2\text{-}COO^-$$

Figure 6 - Structures of organic modifiers for capillary surface modification

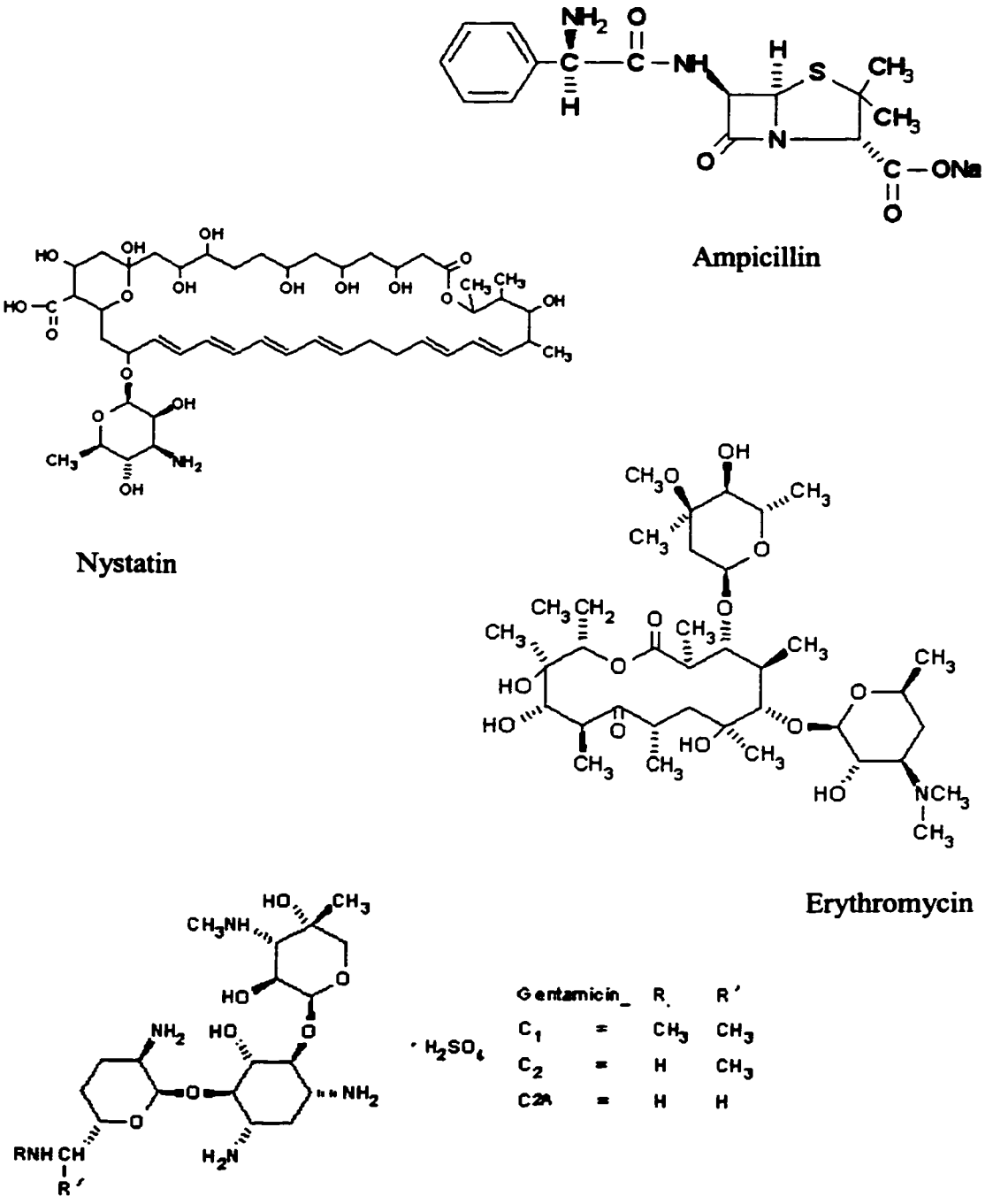


Figure 7 - Structures of ampicillin, nystatin, erythromycin, and gentamycin (Sigma Corporation Online Catalog).

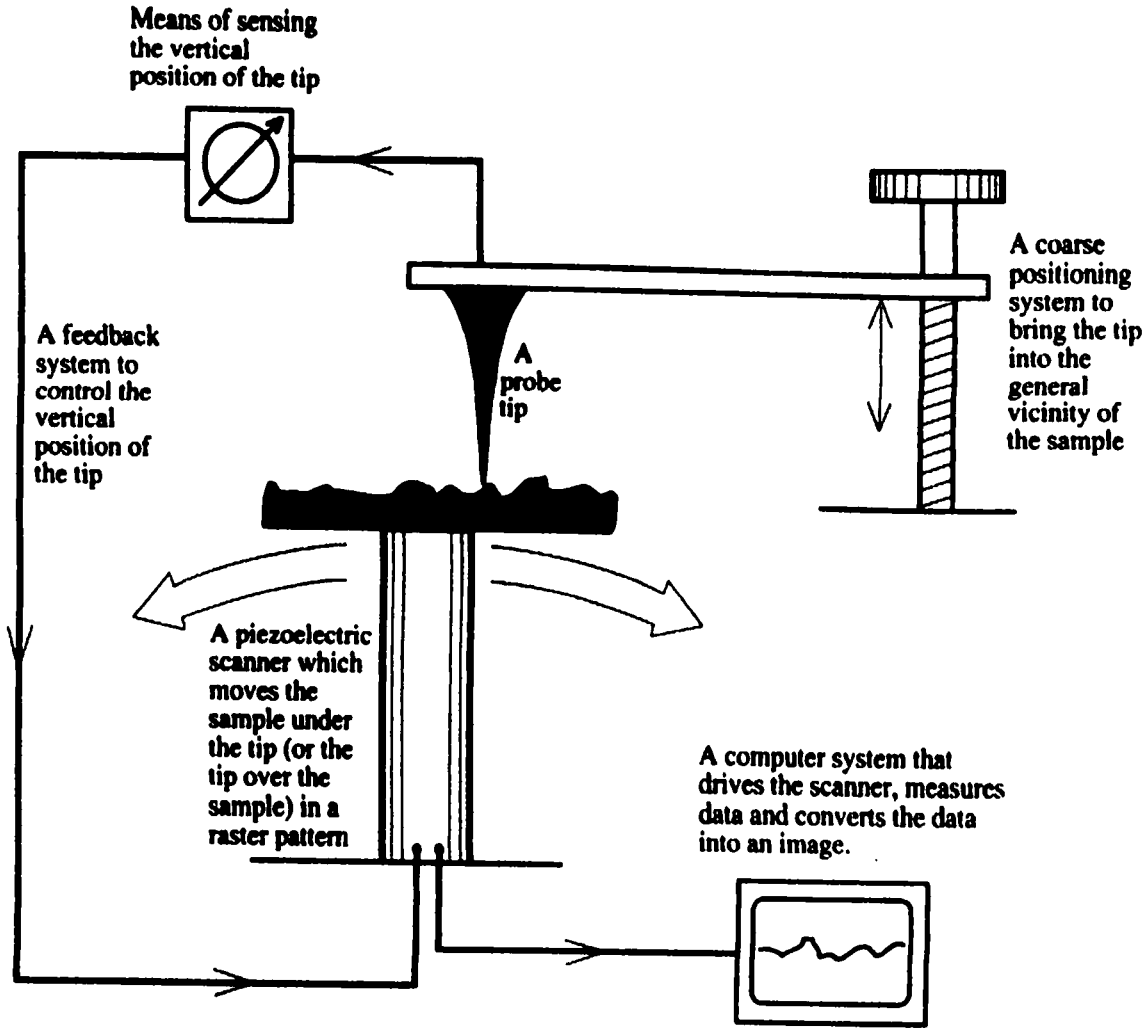


Figure 8 - Scheme of a scanning probe microscope (16).

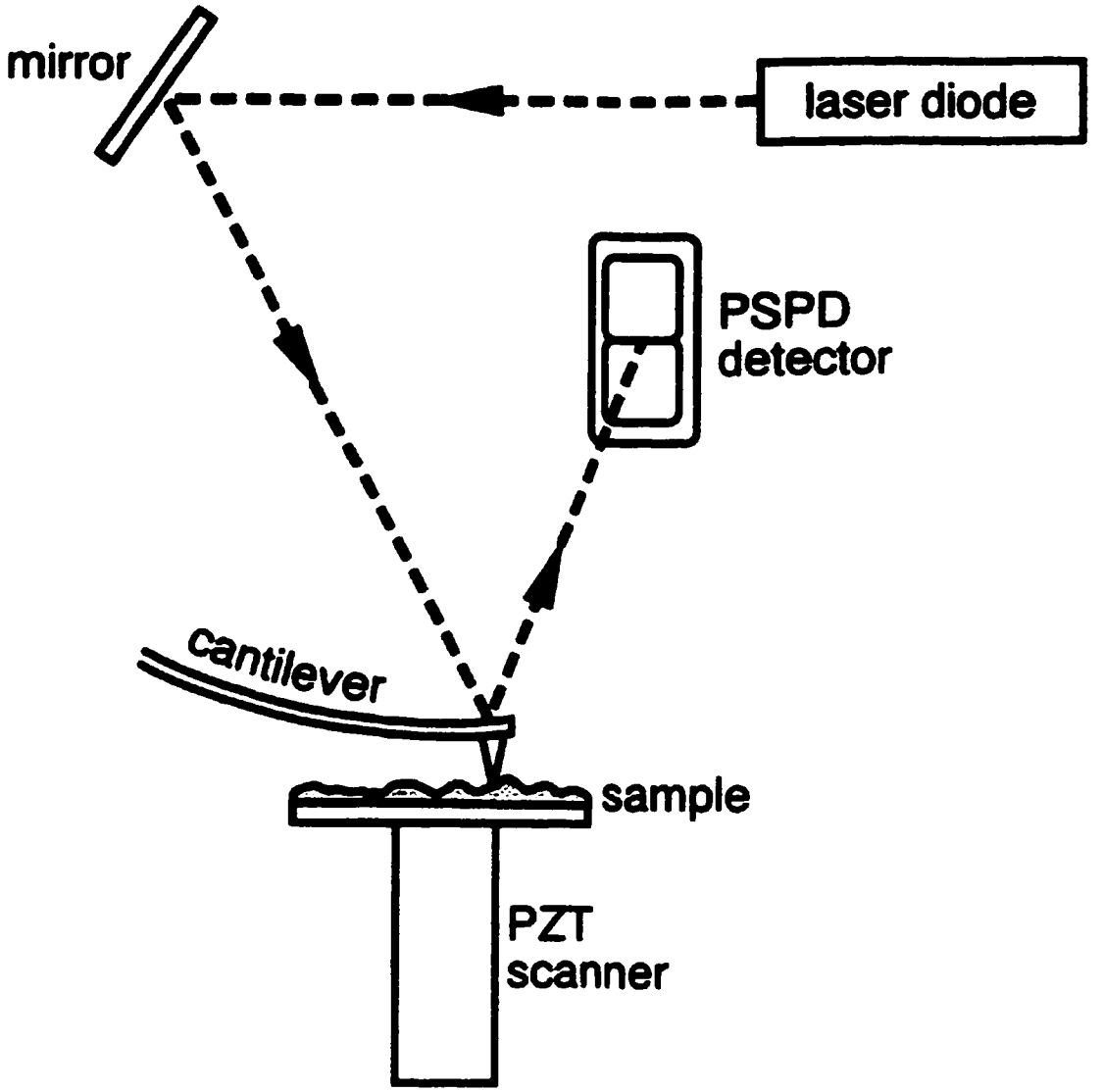


Figure 9 – Beam-bounce detection system (16).

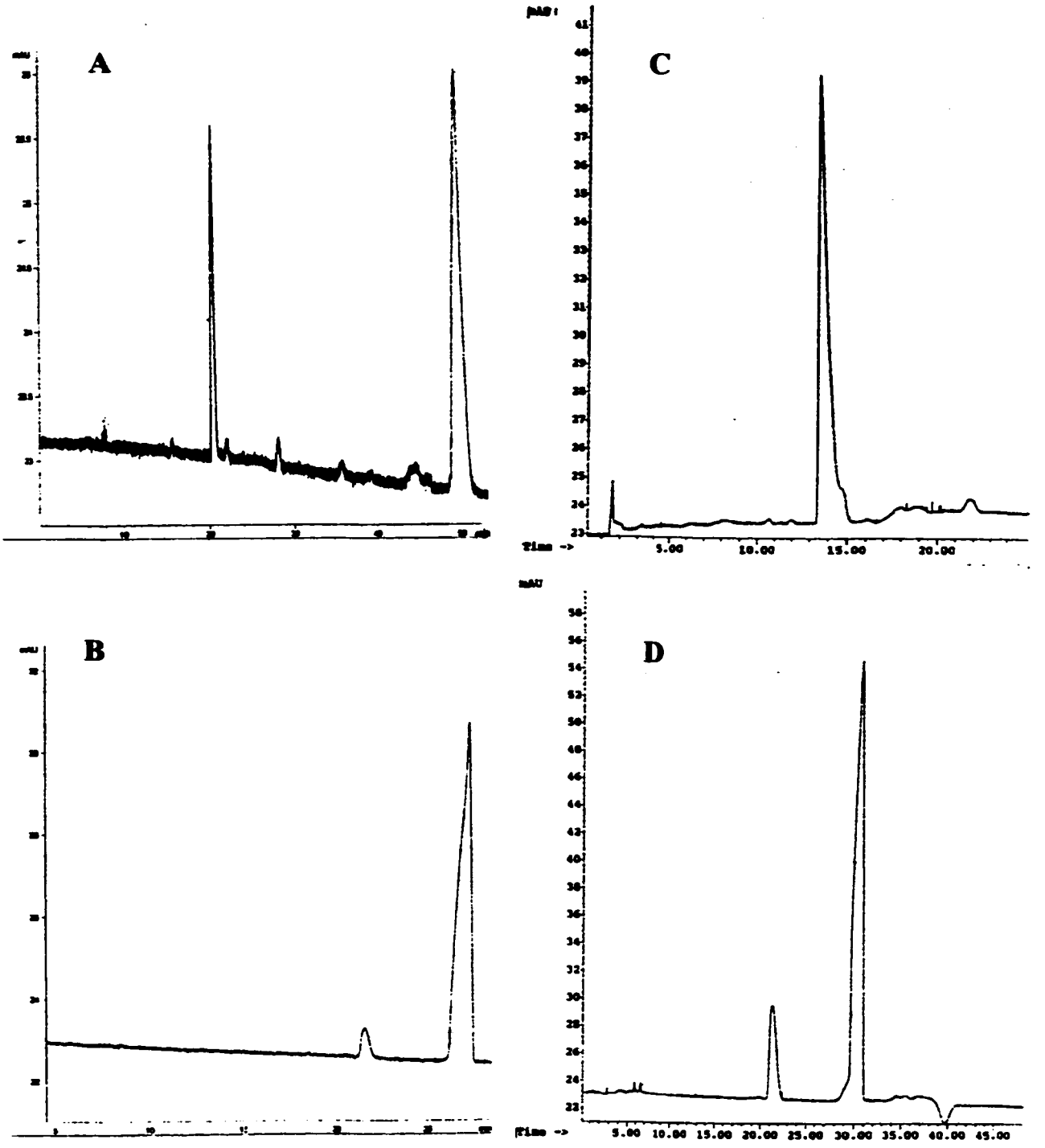


Figure 10 – Separation of ampicillin using 20 μm and 50 μm i.d. C18-modified capillaries at pH 2.14 and 4.41. (A) 20 μm: pH 2.14 (1:10), 10 kV, 2 μA, 5 sec, 211 nm. (B) 20 μm (pH 4.41 (1:10), 20 kV, 2 μA, 5 sec, 211 nm. (C) 50 μm: pH 2.14 (1:10), 10 kV, 9 μA, 0.5 sec, 211 nm. (D) 50 μm: pH 4.41 (1:10), 20 kV, 14 μA, 1 sec, 211 nm.

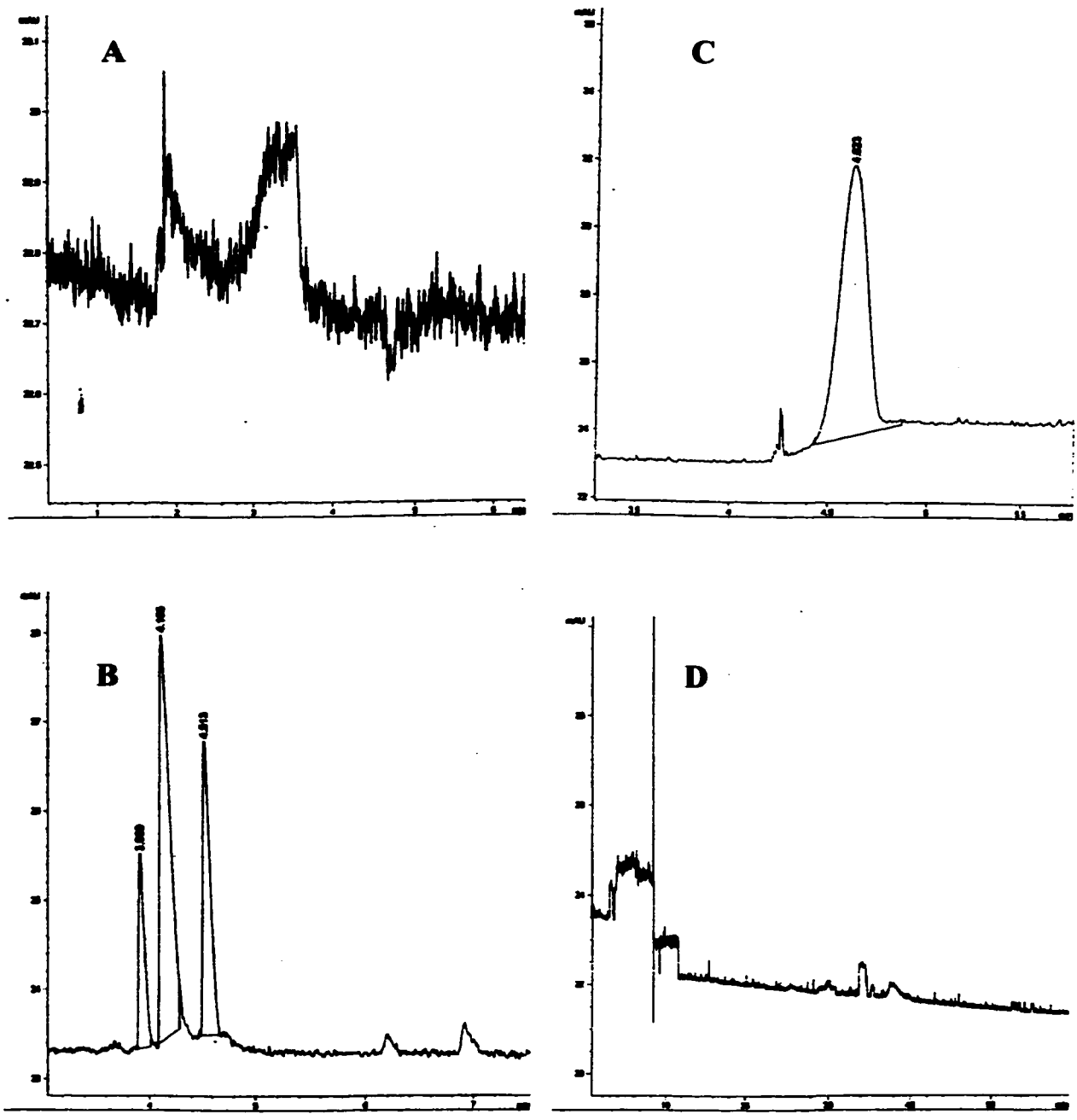


Figure 11 - Separation of ampicillin using 20 μm and 50 μm i. d. C18-modified capillaries at pH 7.40 and 8.14 (1:10). (A) 20 μm: pH 7.40, 20 kV, 1 μA, 5 sec, 211 nm. (B) 20 μm (pH 8.14 (1:5), 30 kV, 1 μA, 1 sec, 211 nm. (C) 50 μm: pH 7.40 (1:10), 20 kV, 5 μA, 5 sec, 215 nm. (D) 50 μm: pH 8.14 (1:10), 20 kV, 2 μA, 1 sec, 215 nm.

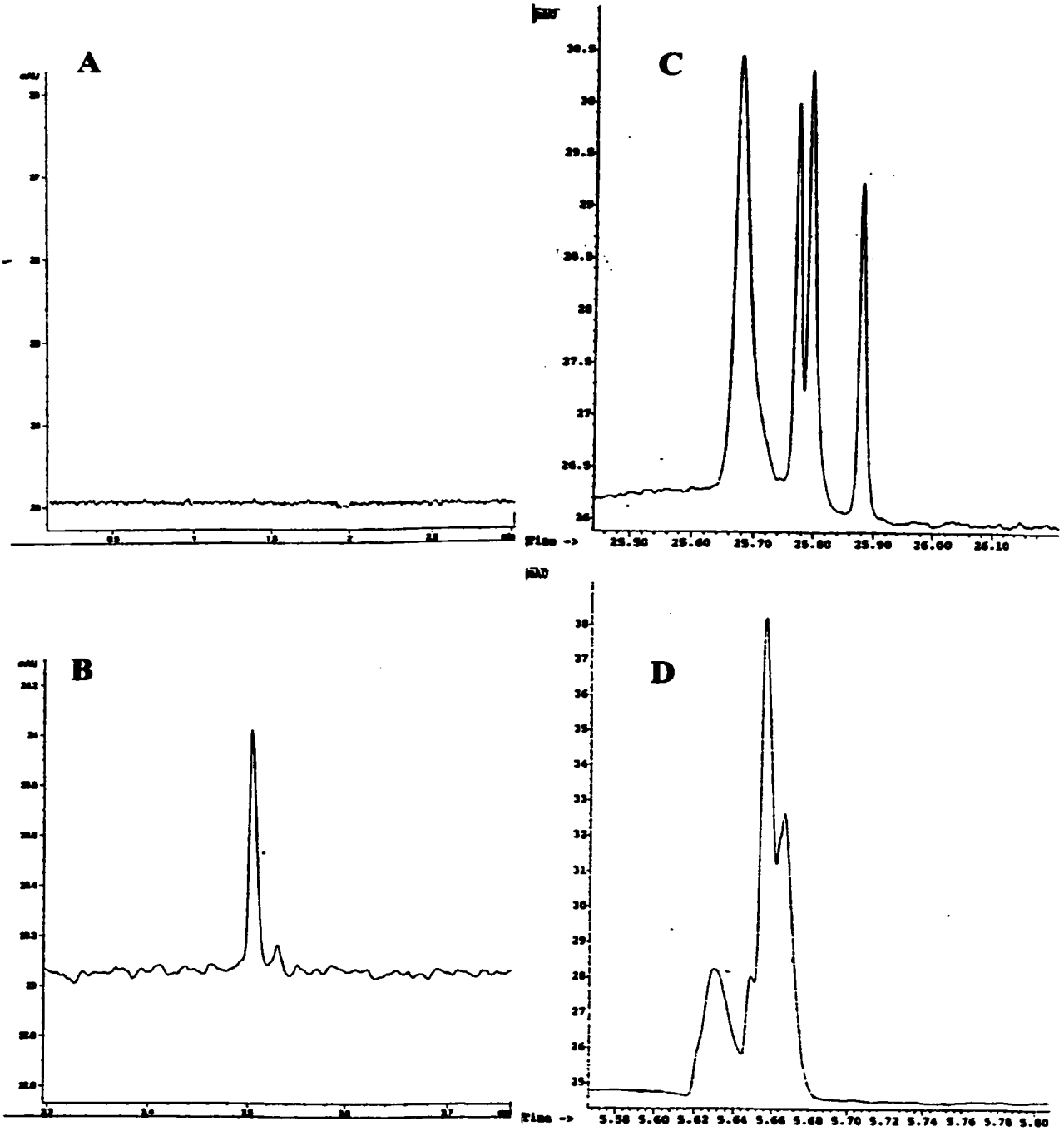


Figure 12 - Separation of gentamycin using 20 μm and 50 μm i. d. C18-modified capillaries at pH 2.14 and 4.41. (A) 20 μm: pH 2.14 (1:10), 0 kV, 0 μA, 5 sec, 20" vac., 254 nm. (B) 20 μm: pH 4.41 (1:10), 20 kV, 2 μA, 5 sec, 254 nm. (C) 50 μm: pH 2.14 (1:20), 5 kV, 4 μA, 10 sec, 254 nm. (D) 50 μm: pH 4.41 (1:10), 20 kV, 16 μA, 20 sec, 215 nm.

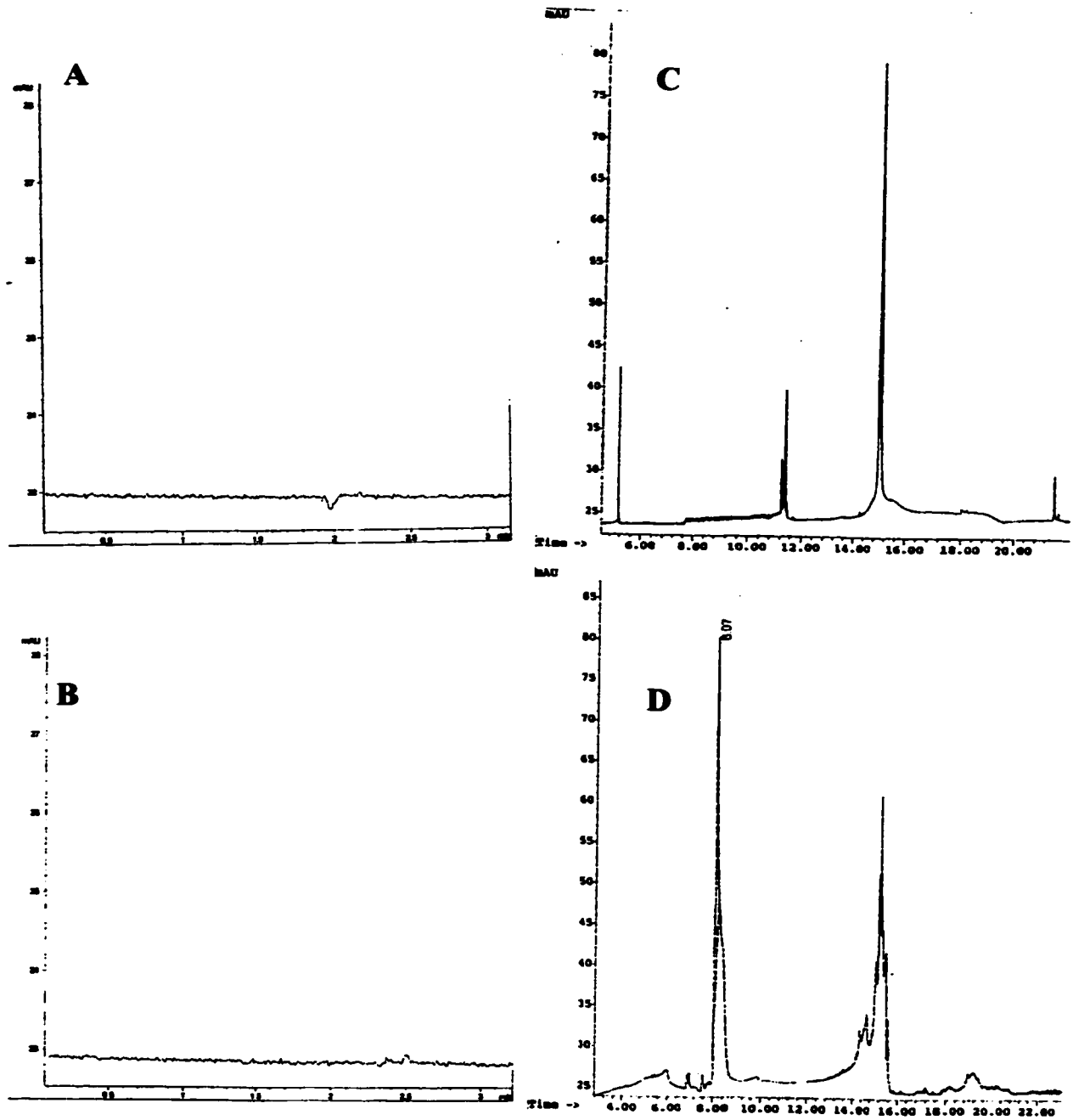


Figure 13 – Separation of gentamycin using 20 μm and 50 μmin i. d. C18-modified capillaries at pH 7.40 and 8.14. (A) 20 μm: pH 7.40 (1:10), 0 kV, 0 μA, 5 sec, 20" vac., 254 nm. (B) 20 μm: pH 8.14 (1:5), 0 kV, 0 μA, 1 sec, 254 nm. (C) 50 μm: pH 7.40 (1:10), 20 kV, 4 μA, 20 sec, 254 nm. (D) 50 μm: pH 8.14 (1:5), 20 kV, 4 μA, 5 sec, 254 nm.

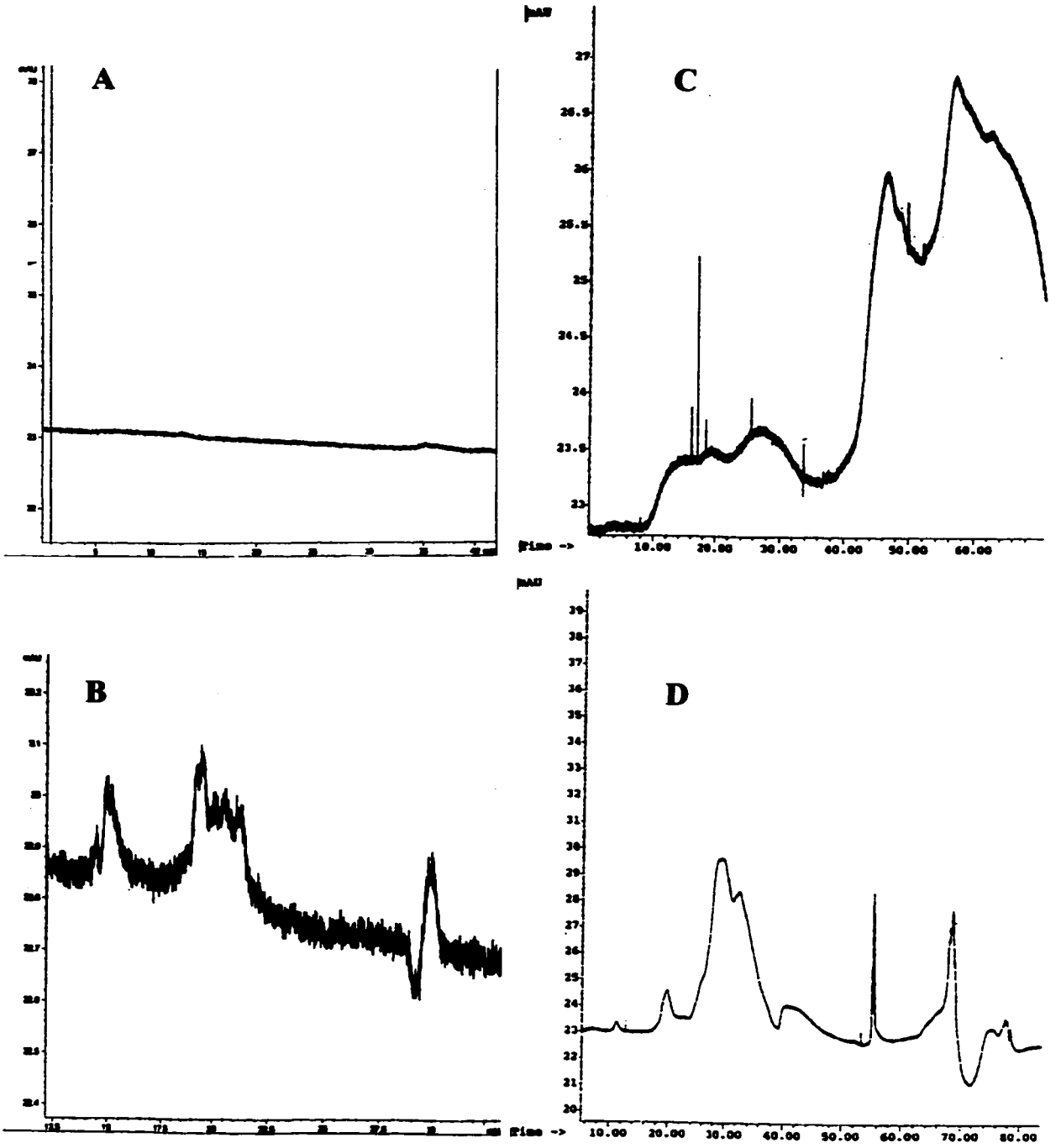


Figure 14 - Separation of nystatin using 20 μm and 50 μmin i. d. C18-modified capillaries at pH 2.14 and 4.41. (A) 20 μm: pH 2.14 (1:10), 20 kV, 5 μA, 5 sec, 254 nm. (B) 20 μm: pH 4.41 (1:10), 20 kV, 2 μA, 5 sec, 254 nm. (C) 50 μm: pH 2.14 (1:20), 10 kV, 9 μA, 1 sec, 290 nm. (D) 50 μm: pH 4.41 (1:10), 20 kV, 14 μA, 5 sec, 254 nm.

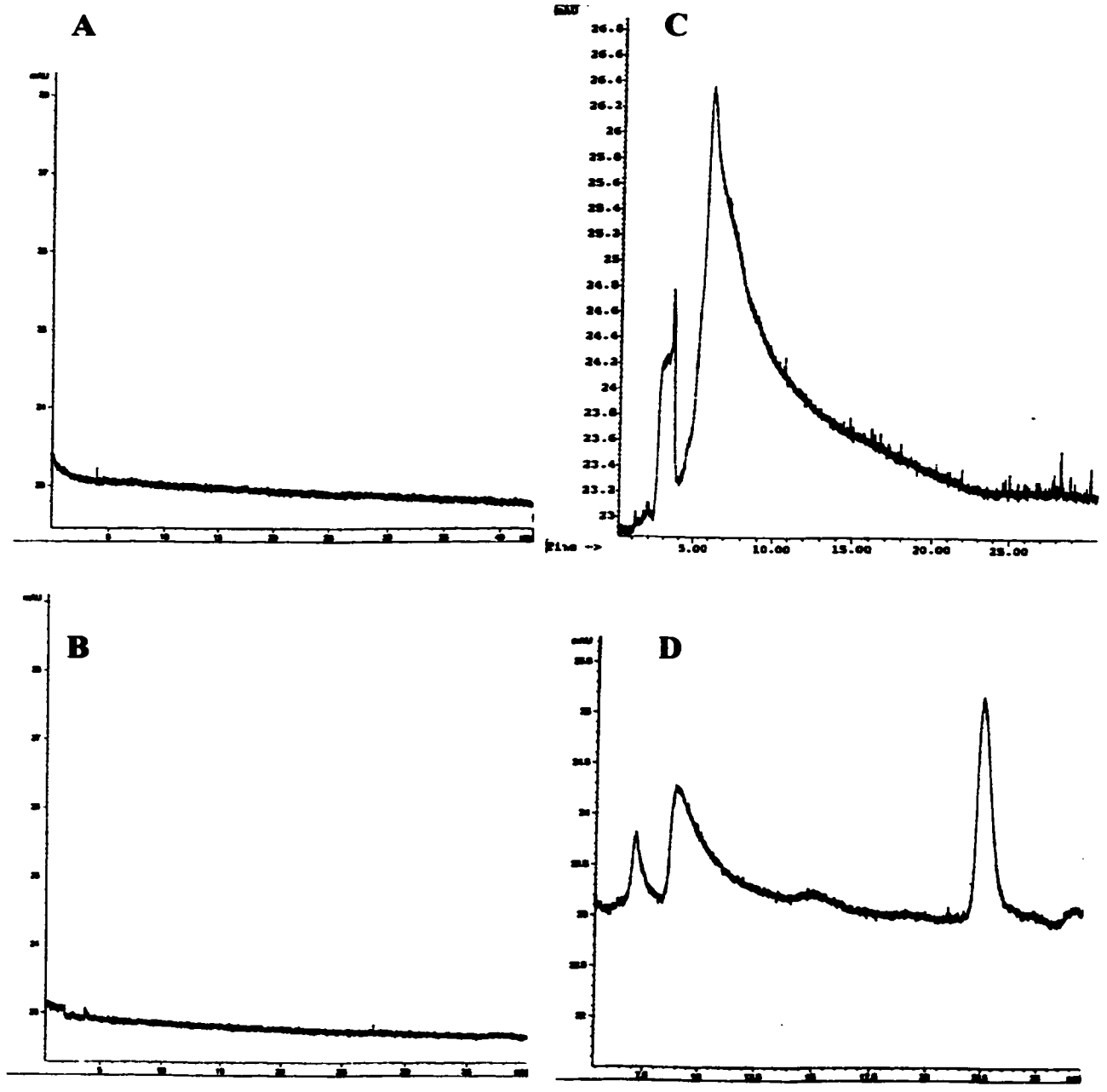


Figure 15 - Separation of nystatin using 20 μm and 50 μmin i. d. C18-modified capillaries at pH 7.40 and 8.14. (A) 20 μm: pH 7.40 (1:10), 20 kV, 1 μA, 7 sec, 215 nm. (B) 20 μm: pH 8.14 (1:5), 30 kV, 1 μA, 5 sec, 215 nm. (C) 50 μm: pH 7.40 (1:10), 20 kV, 4 μA, 20 sec, 290 nm. (D) 50 μm: pH 8.14 (1:10), 20 kV, 2 μA, 5 sec, 300 nm.

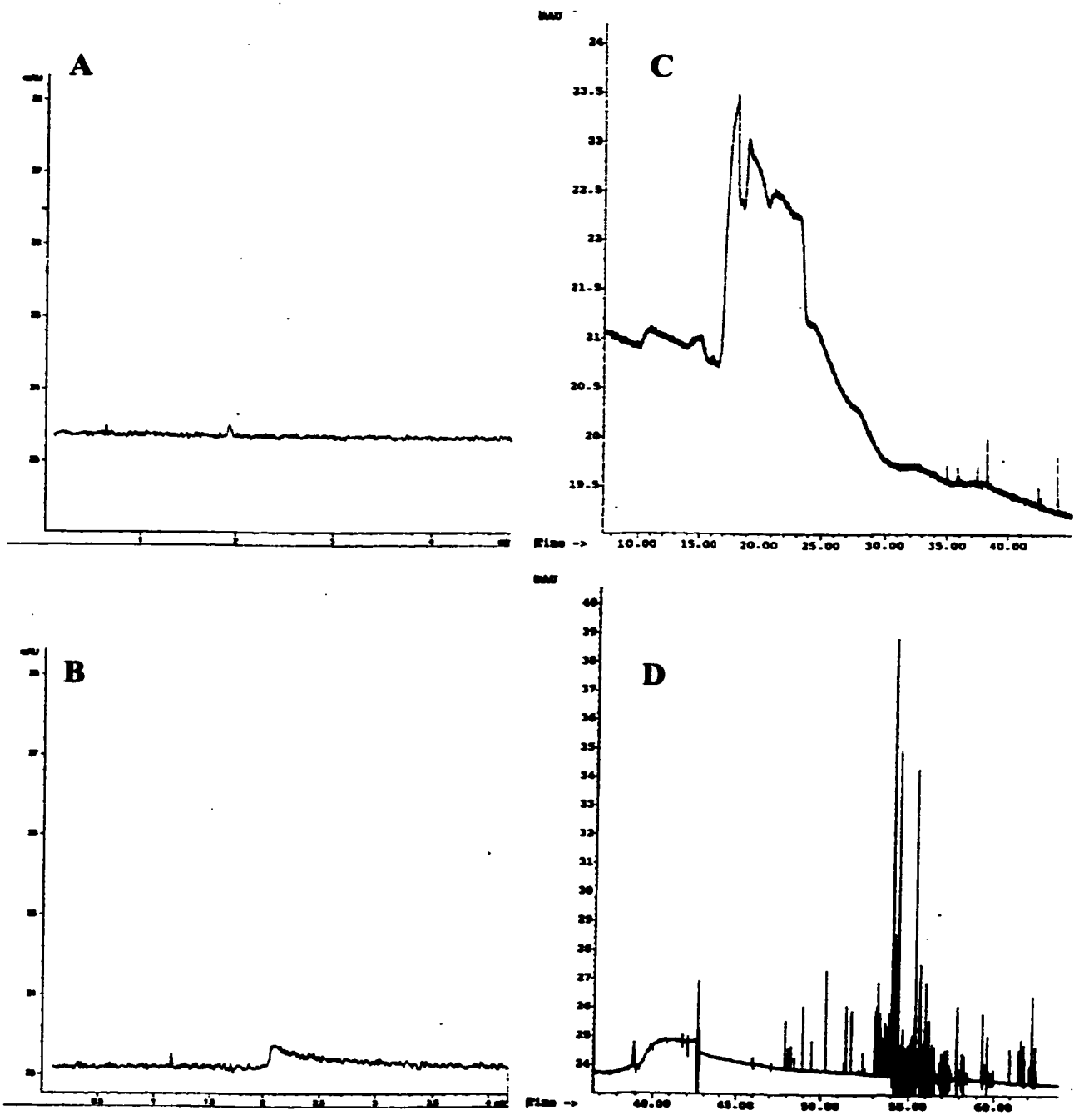


Figure 16 - Separation of erythromycin using 20 μm and 50 μmin i. d. C18-modified capillaries at pH 2.14 and 4.41. (A) 20 μm: pH 2.14 (1:10), 0 kV, 0 μA, 5 sec, 20" vac., 215 nm. (B) 20 μm: pH 4.41 (1:10), 0 kV, 0 μA, 1 sec, 20" vac., 211 nm. (C) 50 μm: pH 2.14 (1:20), 10 kV, 9 μA, 10 sec, 211 nm. (D) 50 μm: pH 4.41 (1:10), 20 kV, 15 μA, 20 sec, 211 nm.

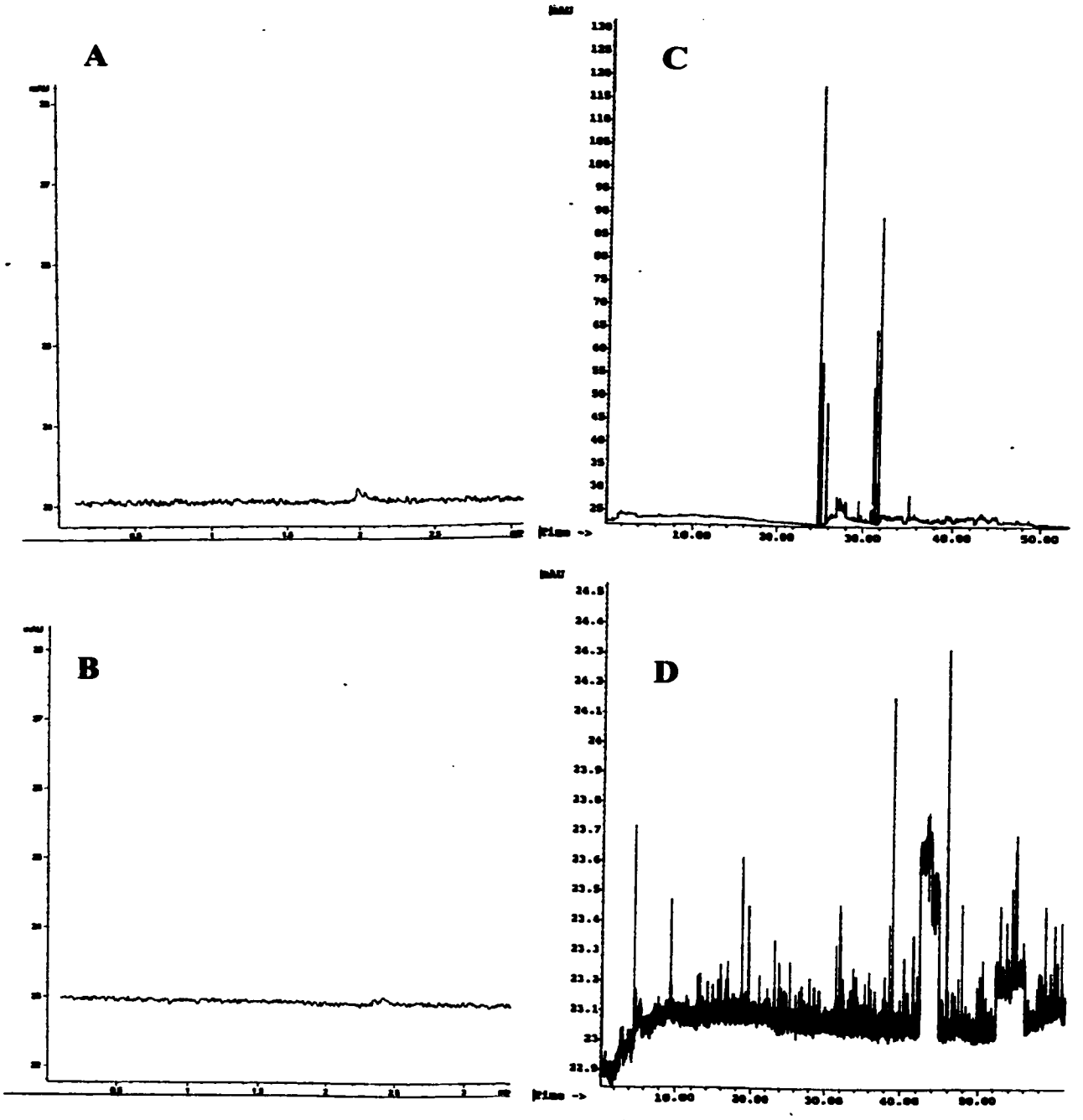


Figure 17 - Separation of erythromycin using 20 μm and 50 μmin i. d. C18-modified capillaries at pH 7.40 and 8.14. (A) 20 μm: pH 7.40 (1:10), 0 kV, 0 μA, 5 sec, 20" vac., 215 nm. (B) 20 μm: pH 8.14 (1:5), 0 kV, 0 μA, 5 sec, 20" vac., 215 nm. (C) 50 μm: pH 7.40 (1:10), 20 kV, 5 μA, 20 sec, 211 nm. (D) 50 μm: pH 8.14 (1:5), 20 kV, 2 μA, 10 sec, 290 nm.

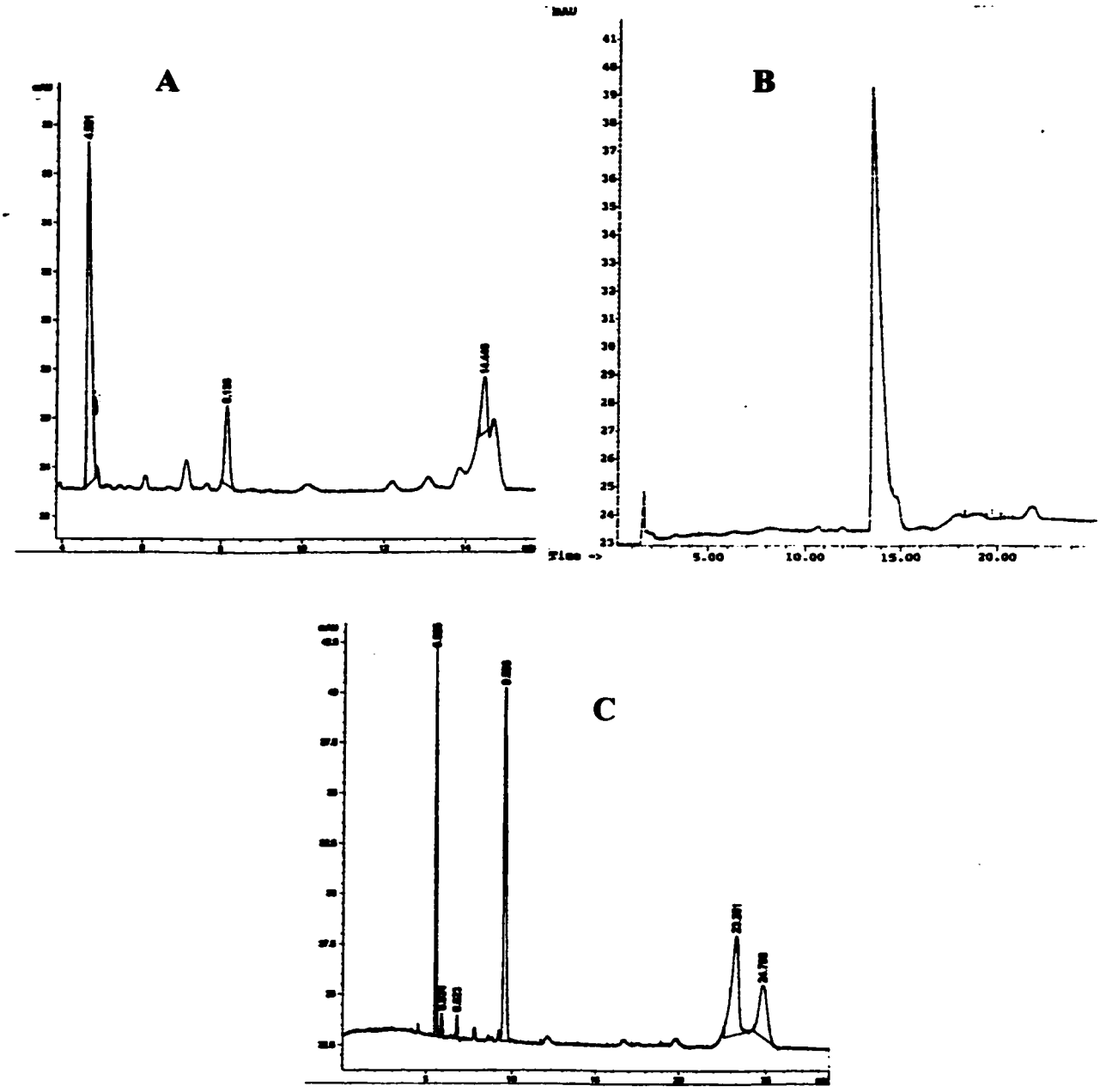


Figure 18 - Separation of ampicillin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 2.14 (1:20). (A) etched: 20 kV, 15 μA, 0.5 sec, 211 nm. (B) C18: 10 kV, 9 μA, 0.5 sec, 211 nm. (C) cholesteryl: 20 kV, 33 μA, 0.5 sec, 211 nm.

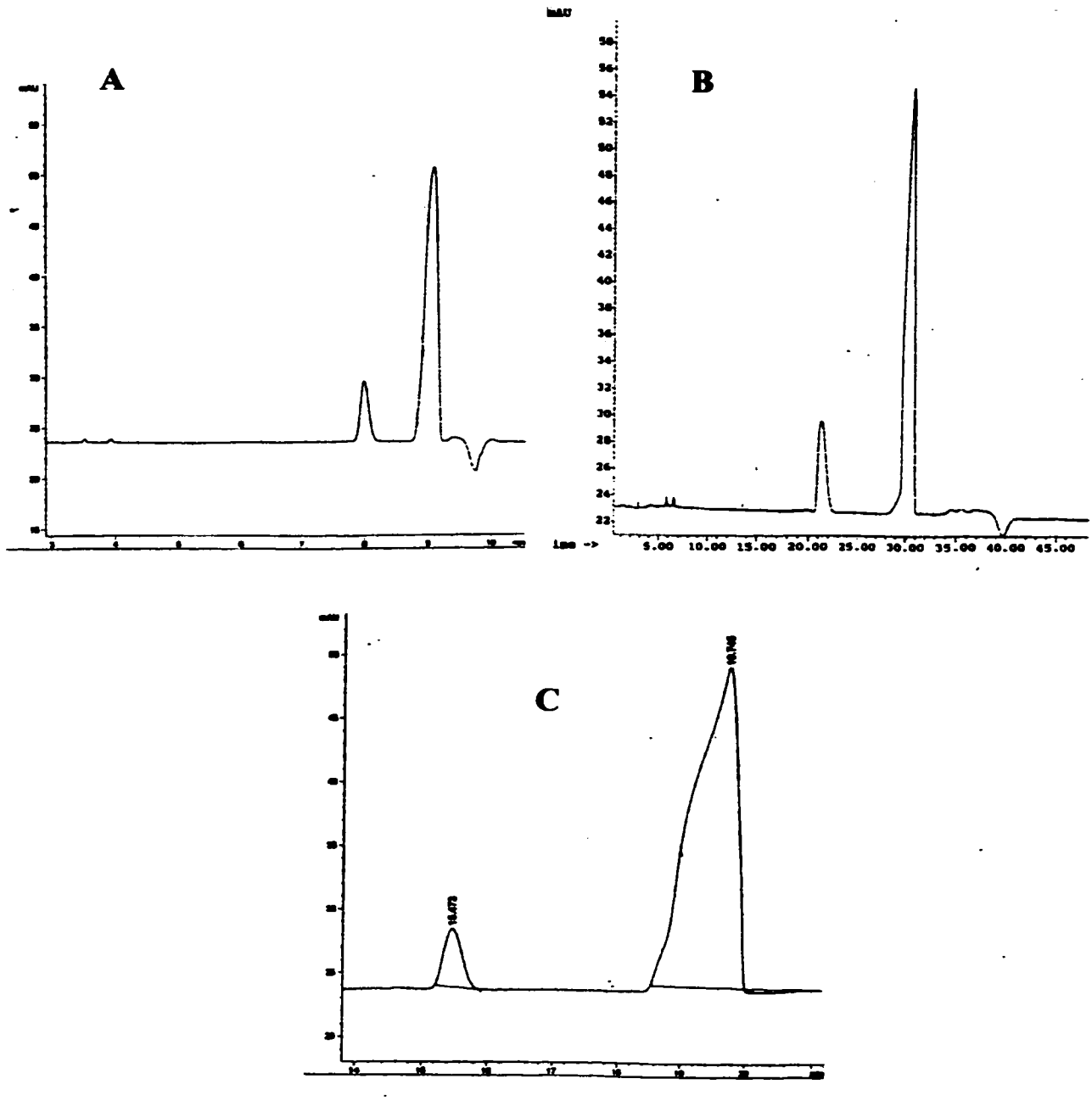


Figure 19 – Separation of ampicillin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 4.41 (1:10). (A) etched: 20 kV, 13 μA, 0.5 sec, 211 nm. (B) C18: 20 kV, 14 μA, 1 sec, 211 nm. (C) cholesteryl: 20 kV, 16 μA, 1 sec, 211 nm.

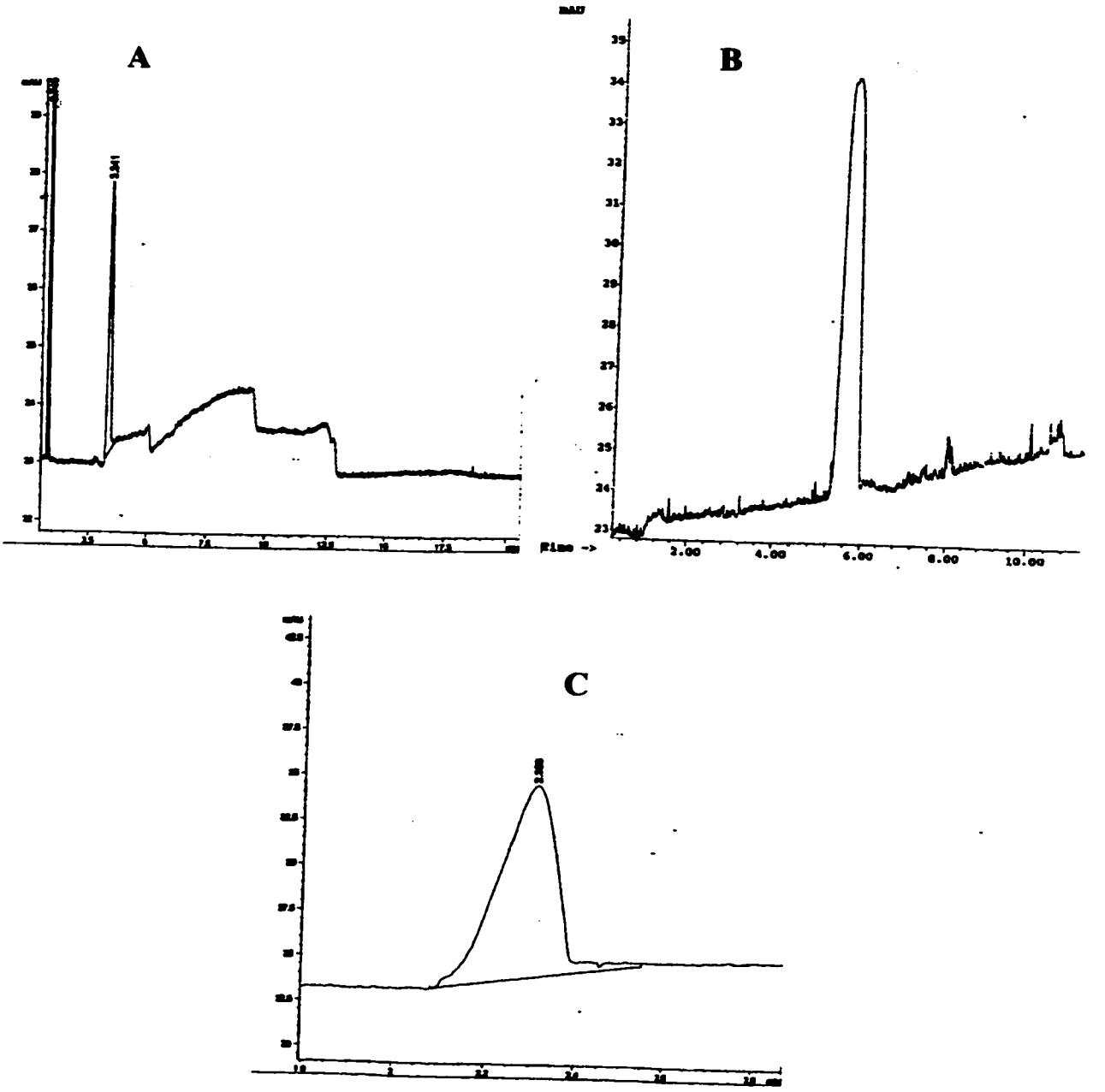


Figure 20 - Separation of ampicillin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 7.40 (1:10). (A) etched: 20 kV, 5 μA, 0.5 sec, 211 nm. (B) C18: 20 kV, 5 μA, 5 sec, 211 nm. (C) cholesteryl: 20 kV, 6 μA, 5 sec, 211 nm.

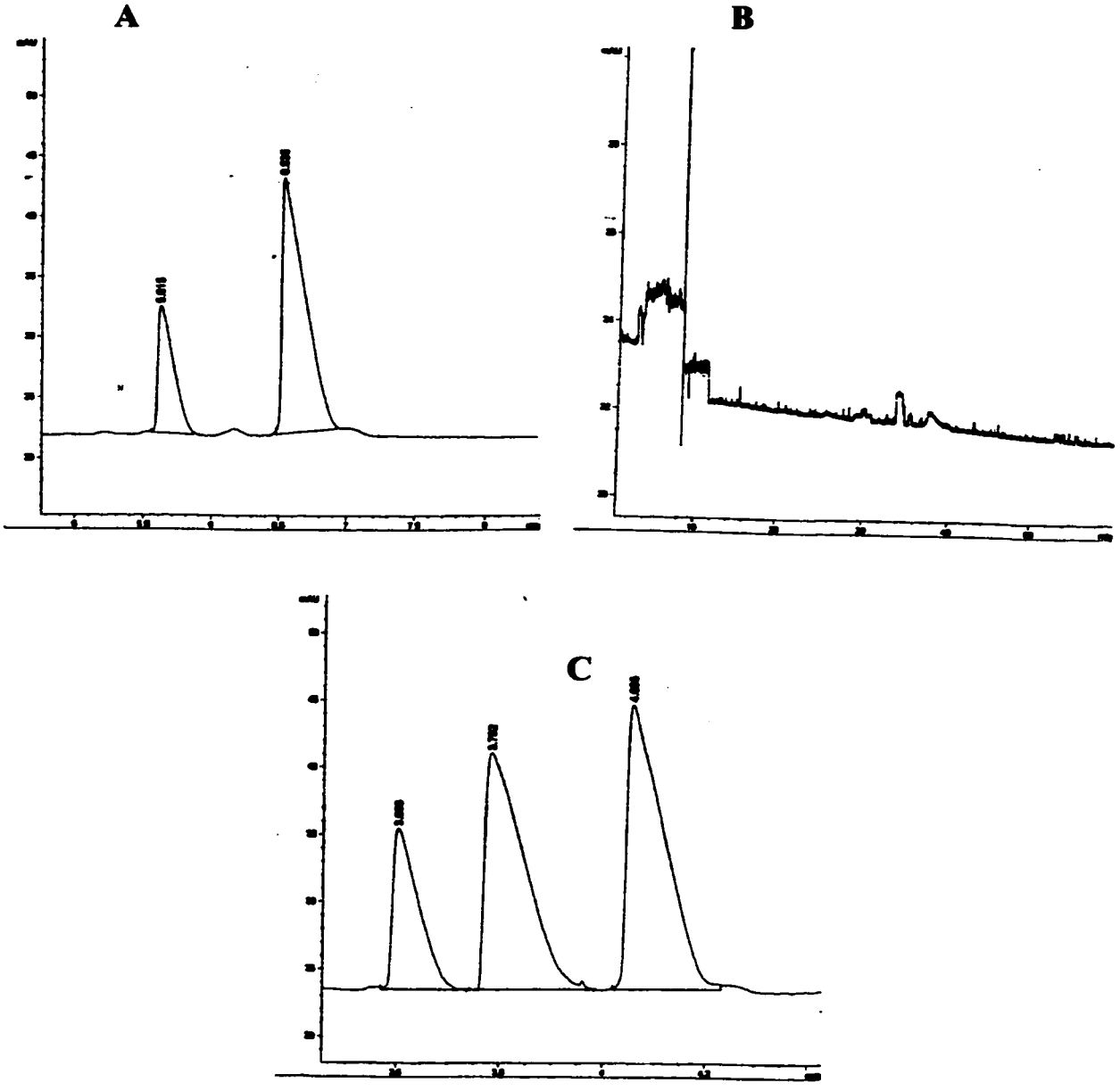


Figure 21 - Separation of ampicillin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 8.14. (A) etched (1:5): 20 kV, 2 μA, 0.5 sec, 211 nm. (B) C18 (1:10): 20 kV, 2 μA, 1 sec, 215 nm. (C) cholesteryl (1:10): 20 kV, 16 μA, 0.5 sec, 211 nm.

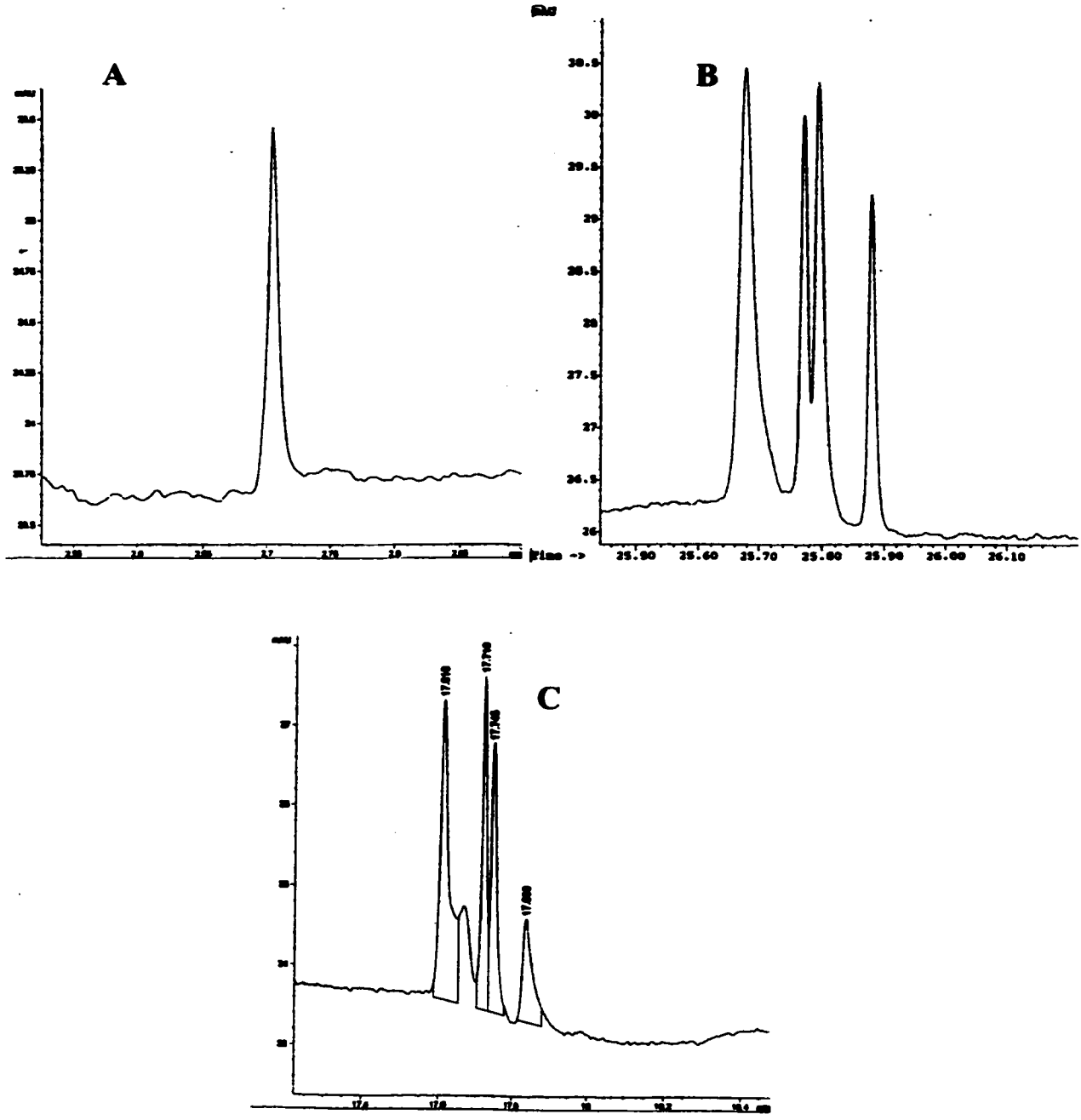


Figure 22 - Separation of gentamycin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 2.14. (A) etched (1:20): 20 kV, 15 μA, 10 sec, 254 nm. (B) C18 (1:20): 5 kV, 4 μA, 10 sec, 254 nm. (C) cholesteryl (1:10): 5 kV, 7 μA, 5 sec, 254 nm.

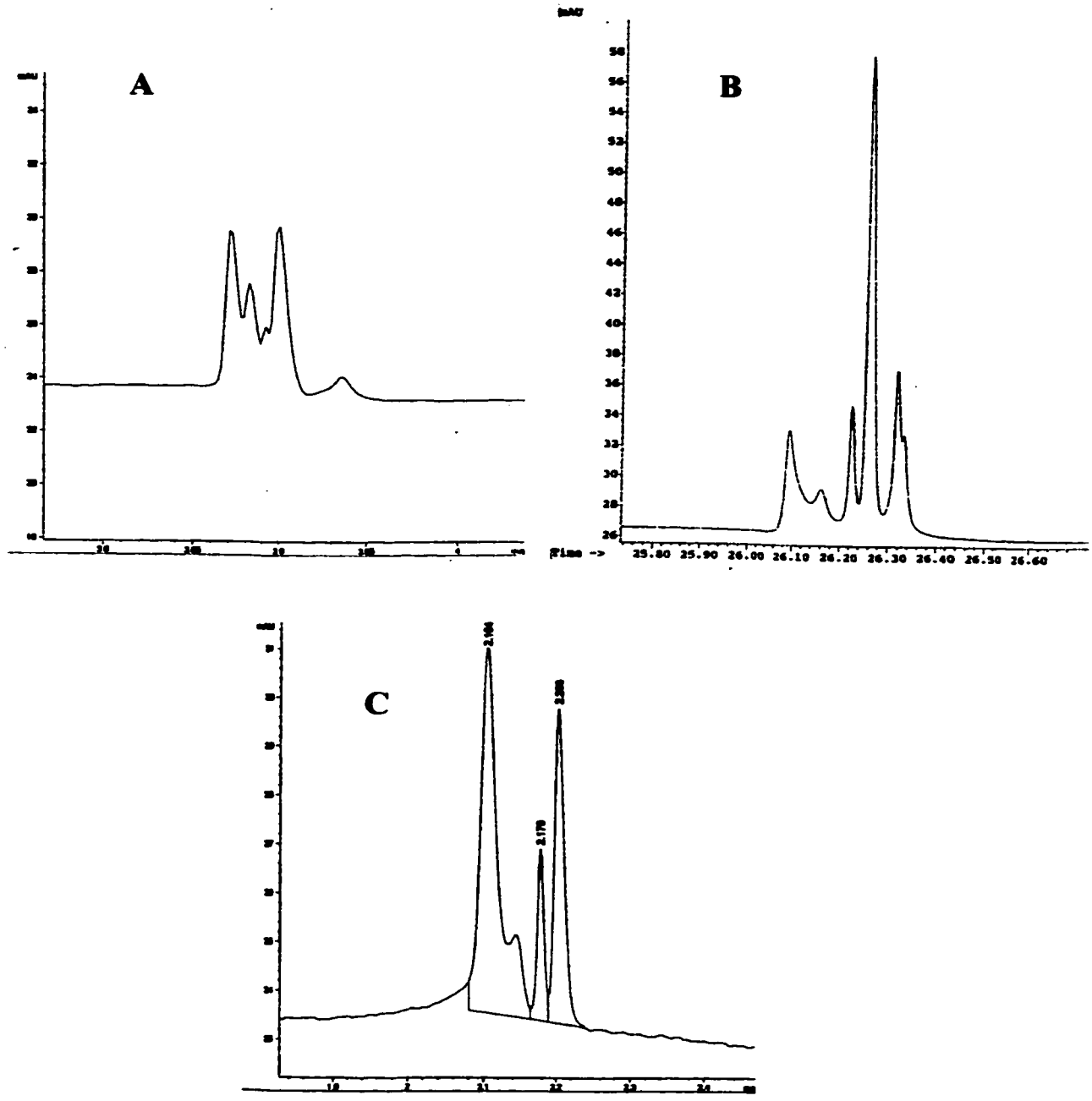


Figure 23 – Separation of gentamycin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 4.41. (A) etched (1:10): 20 kV, 13 μA, 10 sec, 254 nm. (B) C18 (1:20): 5 kV, 3 μA, 20 sec, 254 nm. (C) cholesteryl (1:10): 5 kV, 18 μA, 5 sec, 254 nm.

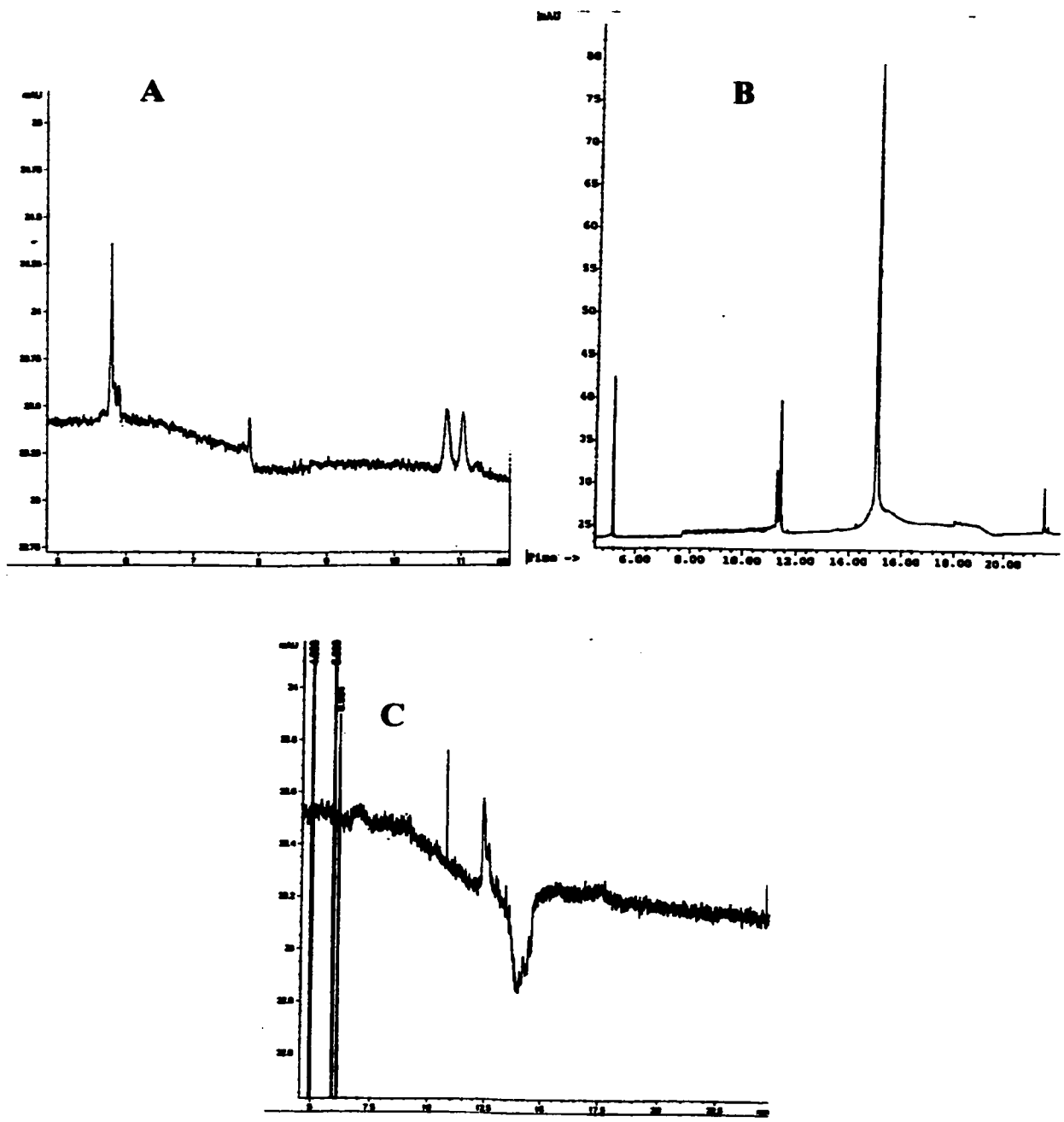


Figure 24 - Separation of gentamycin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 7.40. (A) etched (1:10): 20 kV, 5 μA, 10 sec, 254 nm. (B) C18 (1:20): 20 kV, 4 μA, 20 sec, 254 nm. (C) cholesteryl (1:10): 20 kV, 5 μA, 10 sec, 254 nm.

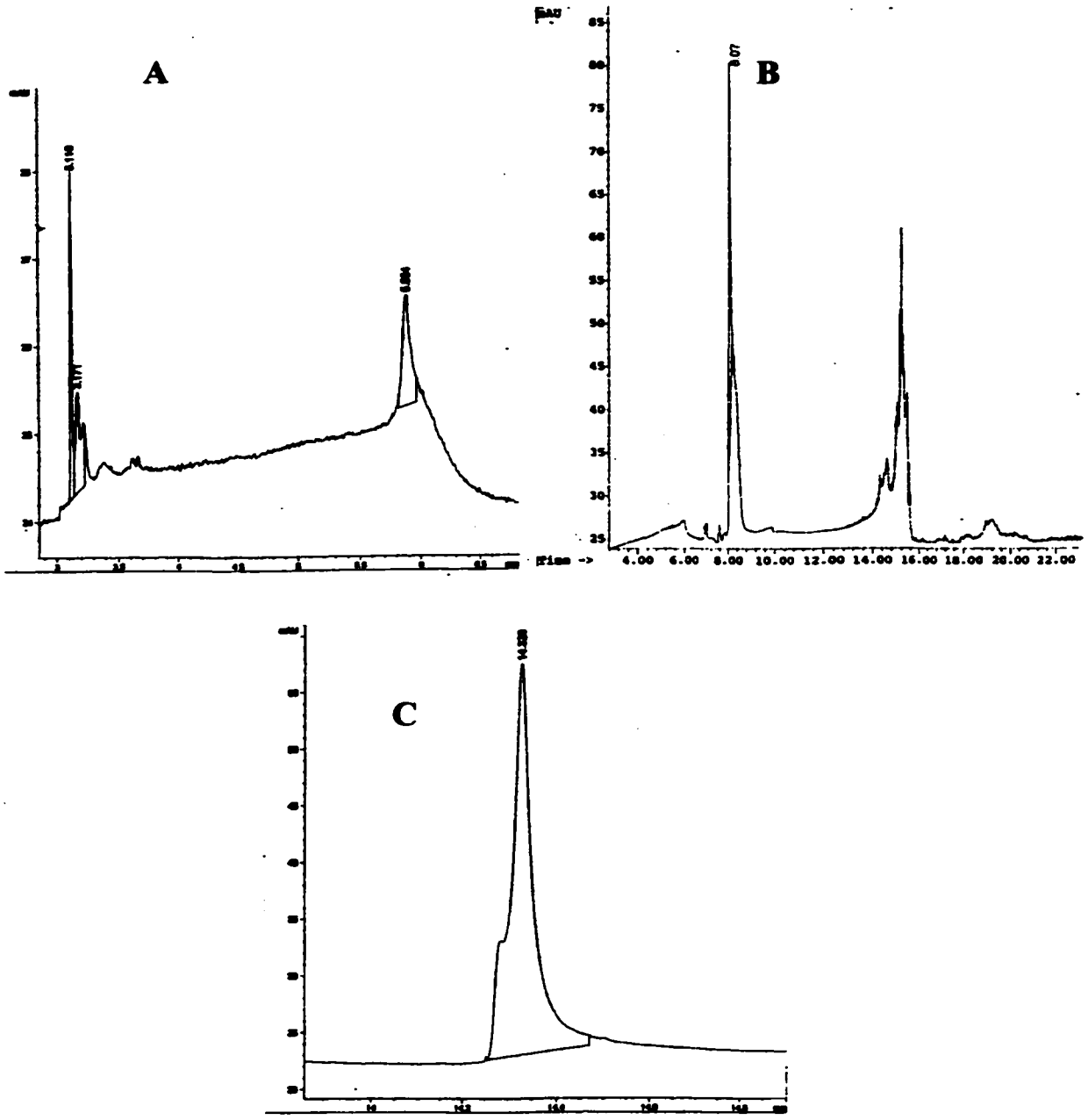


Figure 25 - Separation of gentamycin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 8.14. (A) etched (1:5): 20 kV, 3 μA, 10 sec, 254 nm. (B) C18 (1:5): 20 kV, 4 μA, 5 sec, 254 nm. (C) cholesteryl (1:5): 5 kV, 1 μ , 5 sec, 254 nm.

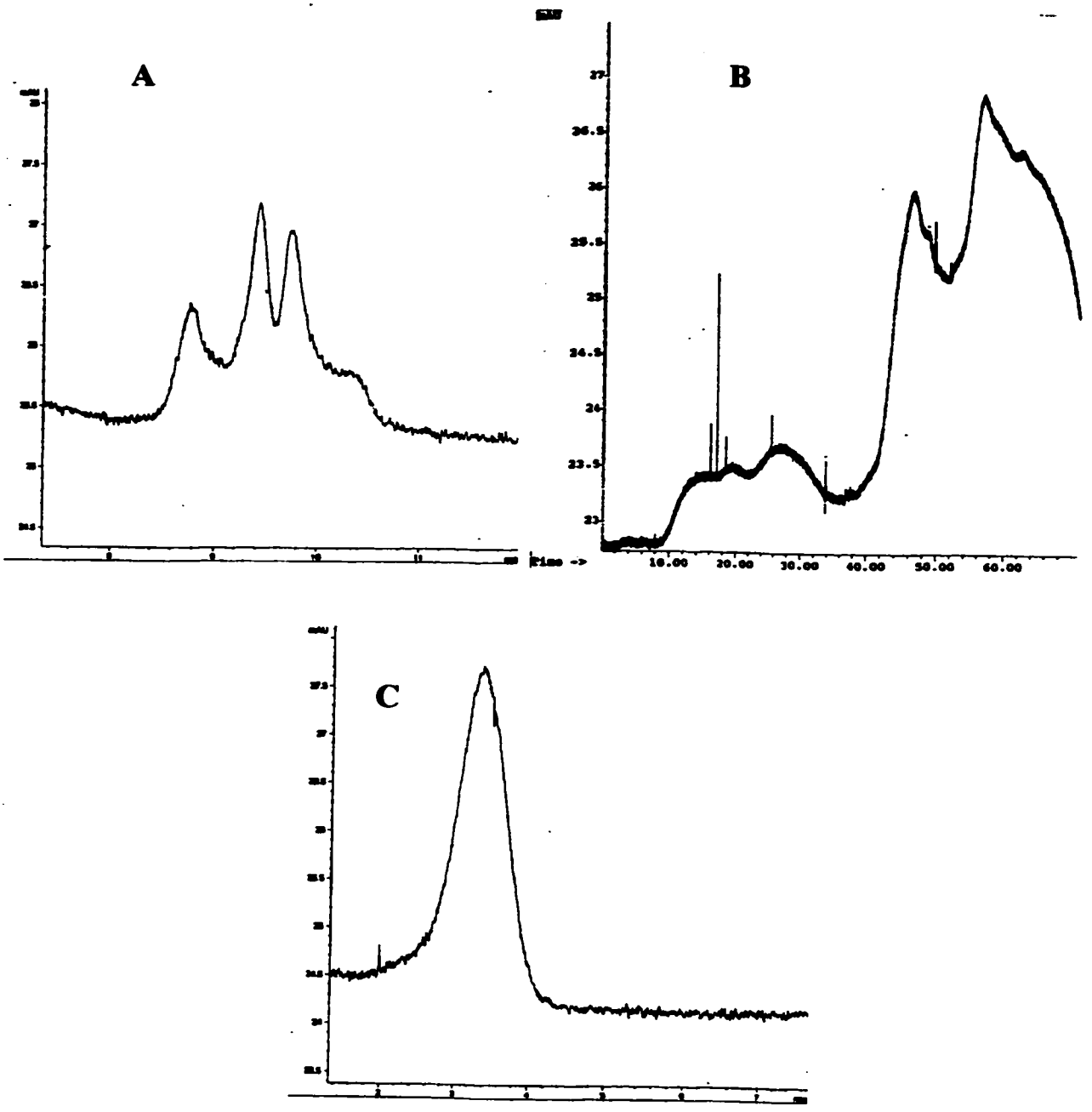


Figure 26 - Separation of nystatin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 2.14. (A) etched (1:20): 20 kV, 15 μA, 1 sec, 211 nm. (B) C18 (1:20): 10 kV, 9 μA, 1 sec, 290 nm. (C) cholesteryl (1:10): 20 kV, 39 μA, 1 sec, 211 nm.

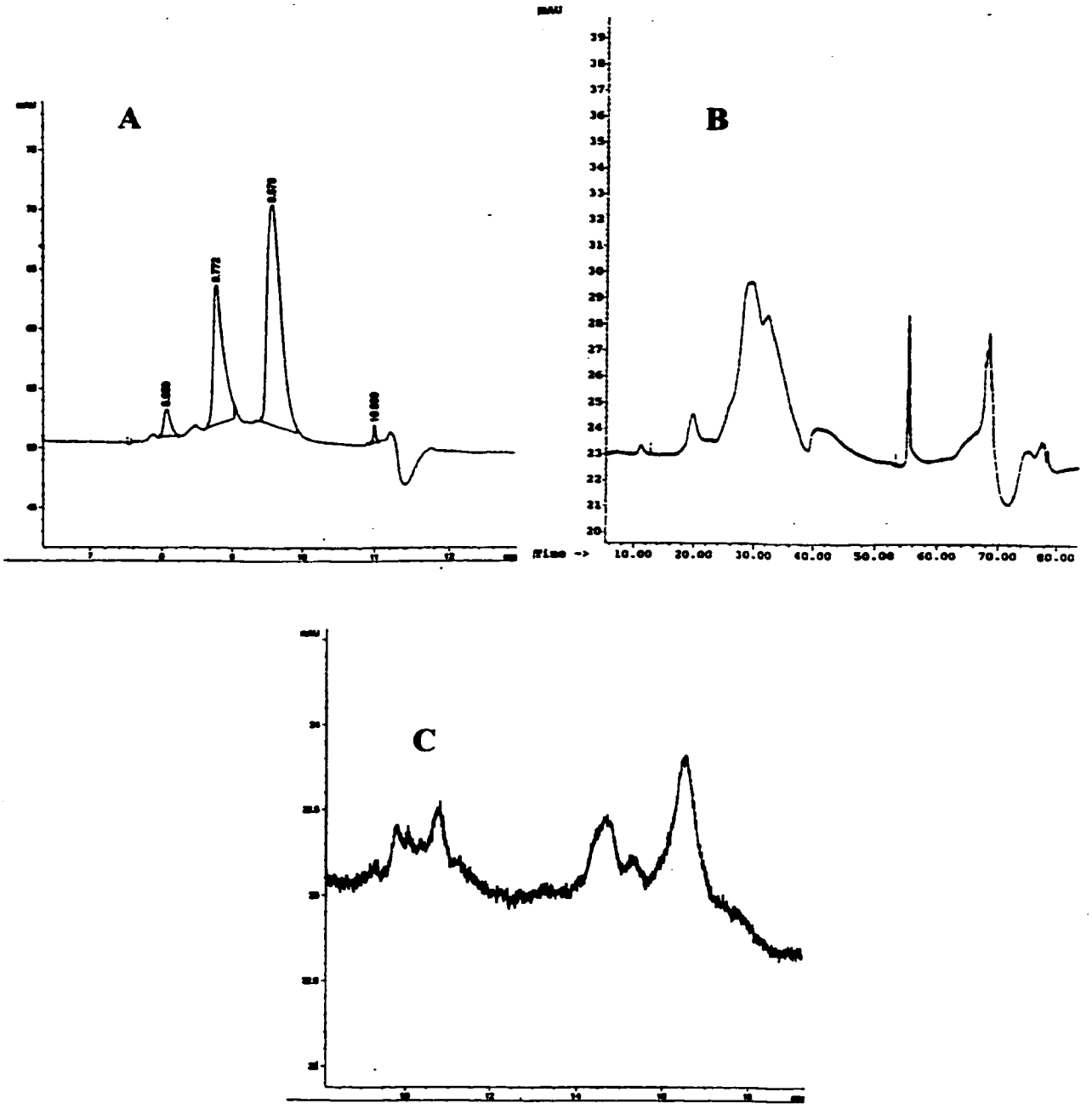


Figure 27 - Separation of nystatin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 4.41. (A) etched (1:10): 20 kV, 11 μA, 2 sec, 290 nm. (B) C18 (1:10): 20 kV, 14 μA, 5 sec, 290 nm. (C) cholesteryl (1:10): 20 kV, 17 μA, 1 sec, 300 nm.

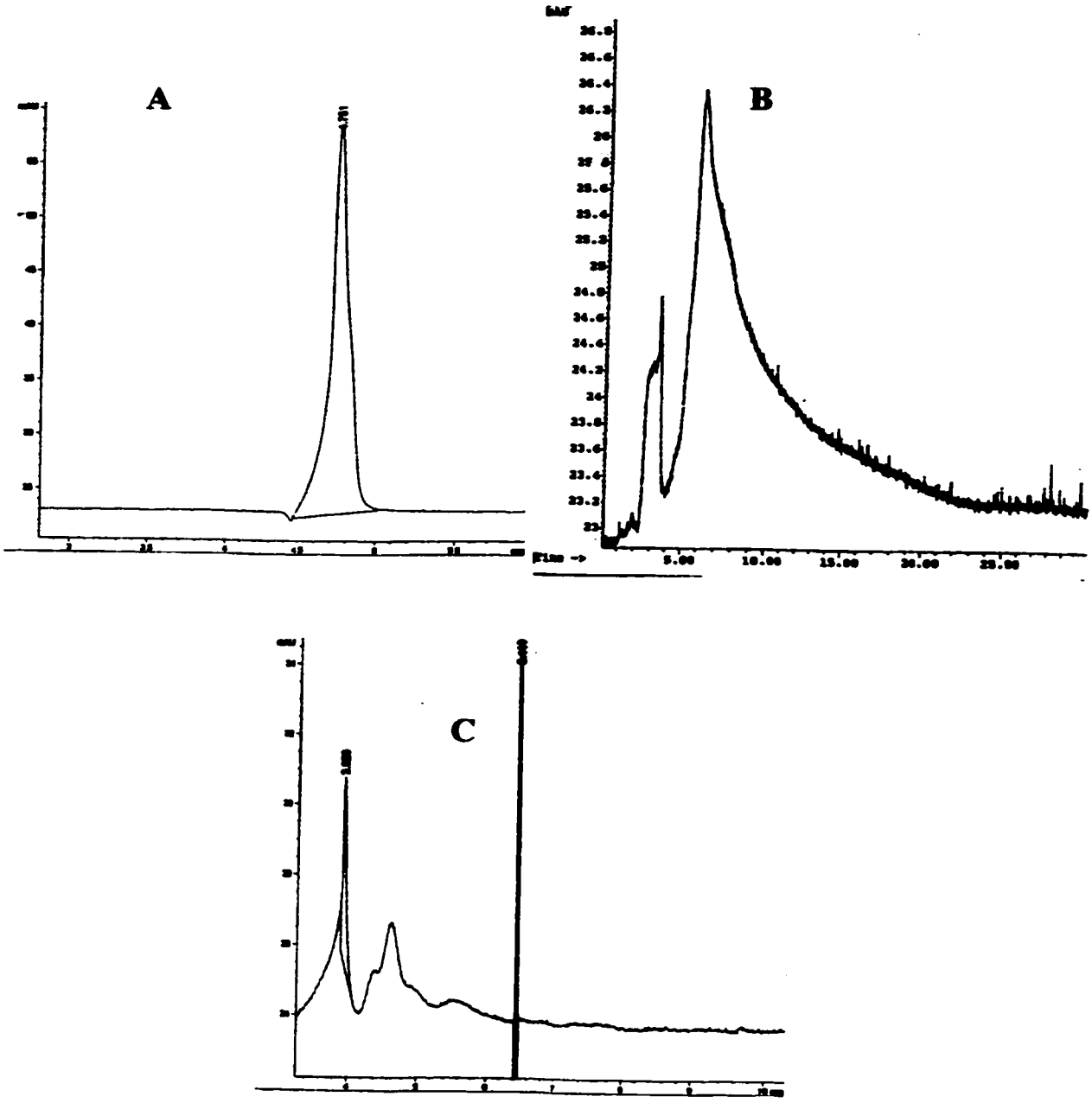


Figure 28 - Separation of nystatin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 7.40. (A) etched (1:10): 20 kV, 5 μA, 2 sec, 290 nm. (B) C18 (1:10): 20 kV, 4 μA, 20 sec, 290 nm. (C) cholesteryl (1:10): 10 kV, 2 μA, 5 sec, 211 nm.

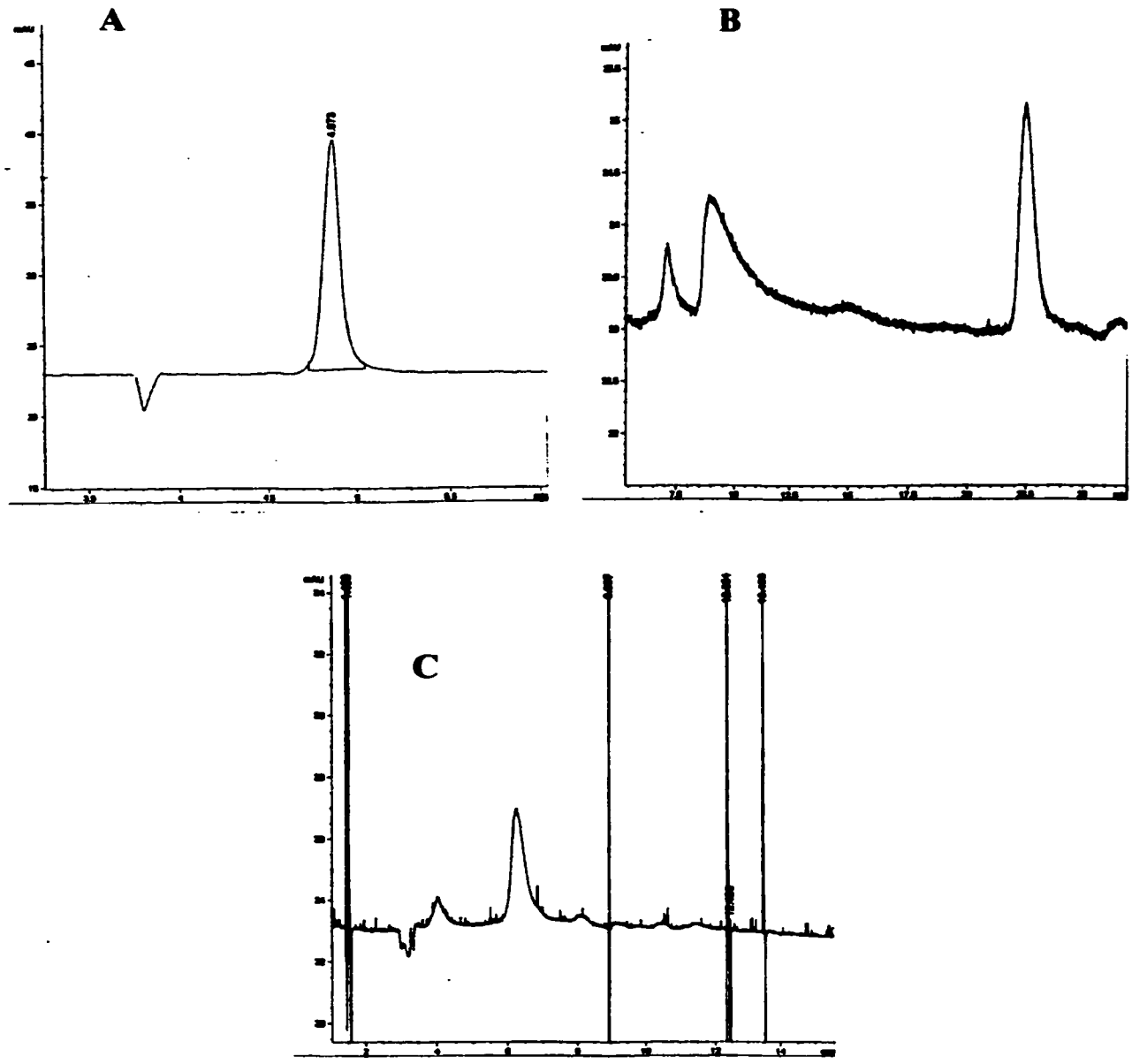


Figure 29 - Separation of nystatin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 8.14. (A) etched (1:5): 20 kV, 2 μA, 1 sec, 290 nm. (B) C18 (1:5): 20 kV, 2 μA, 5 sec, 300 nm. (C) cholesteryl (1:5): 20 kV, 3 μA, 5 sec, 211 nm.

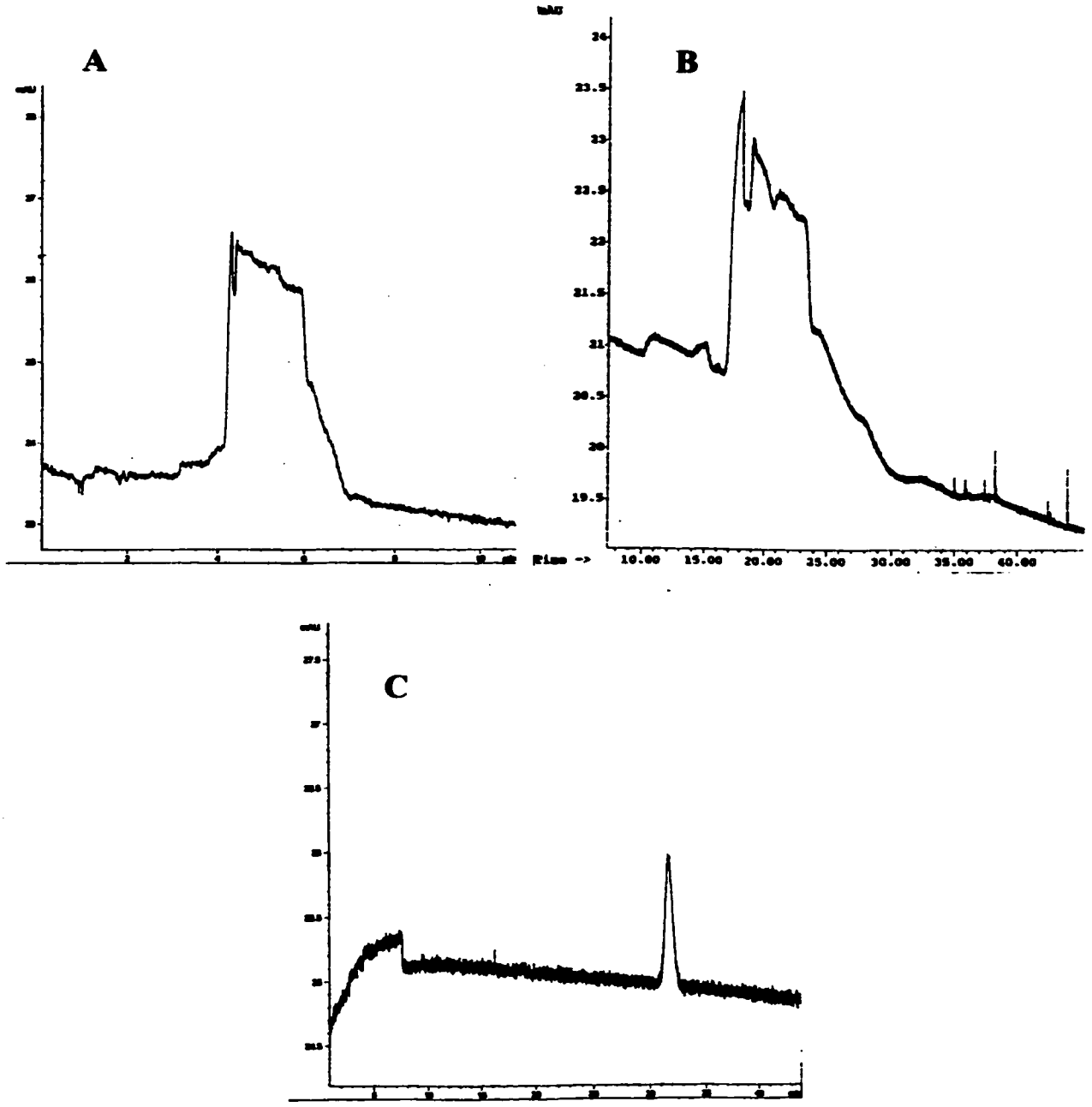


Figure 30 - Separation of erythromycin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 2.14. (A) etched (1:20): 20 kV, 17 μA, 15 sec, 211 nm. (B) C18 (1:20): 10 kV, 9 μA, 10 sec, 290 nm. (C) cholesteryl (1:10): 20 kV, 40 μA, 1 sec, 211 nm.

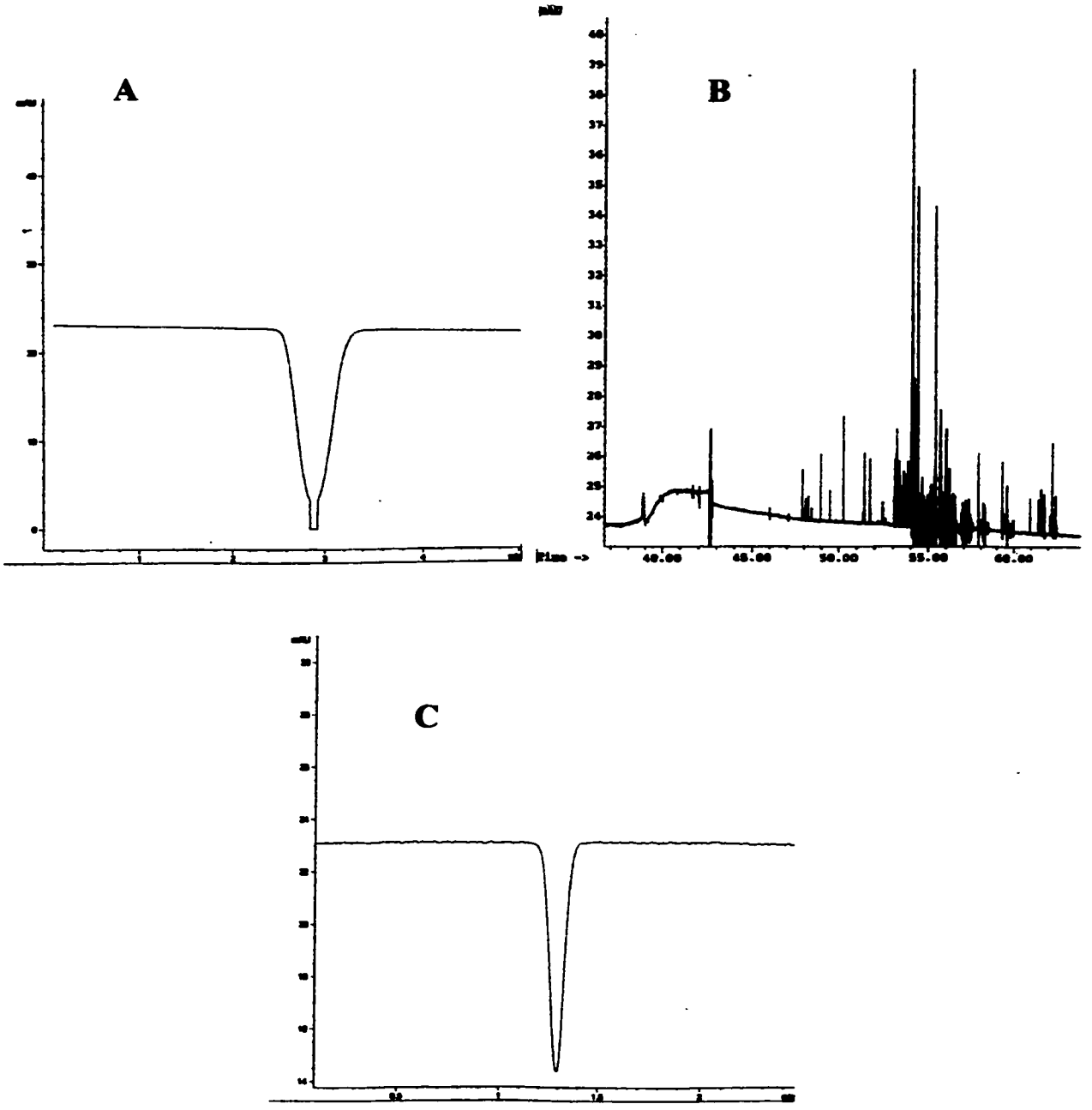


Figure 31 - Separation of erythromycin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 4.41. (A) etched (1:10): 0 kV, 0 μA, 5 sec, 5" vac., 211 nm. (B) C18 (1:10): 20 kV, 15 μA, 20 sec, 211 nm. (C) cholesteryl (1:10): 0 kV, 0 μA, 5 sec, 211 nm.

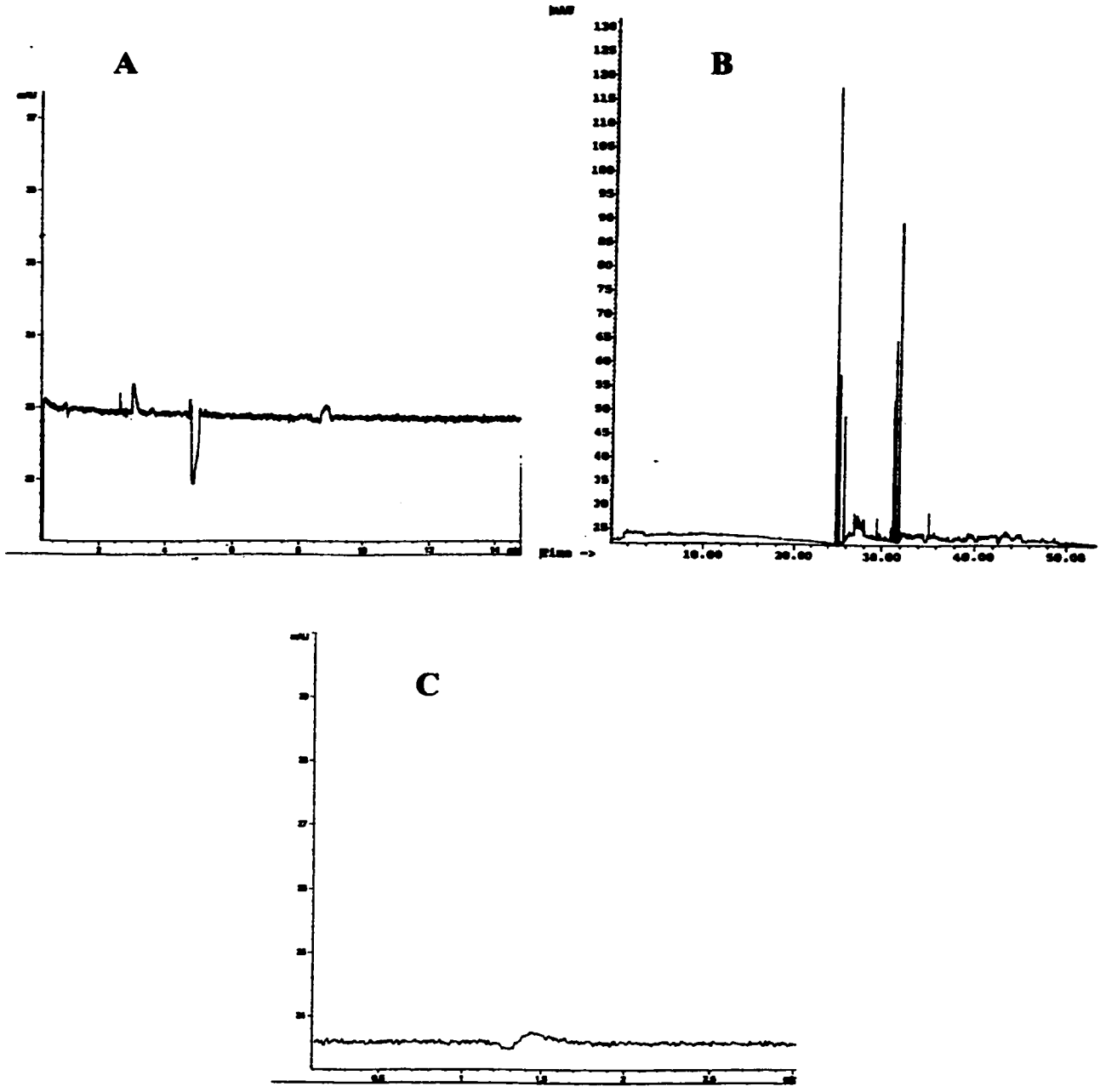


Figure 32 - Separation of erythromycin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 7.40. (A) etched (1:10): 20 kV, 4 μA, 2 sec, 211 nm. (B) C18 (1:10): 20 kV, 5 μA, 20 sec, 211 nm. (C) cholesteryl (1:10): 0 kV, 0 μA , 5" vac., 5 sec, 211 nm.

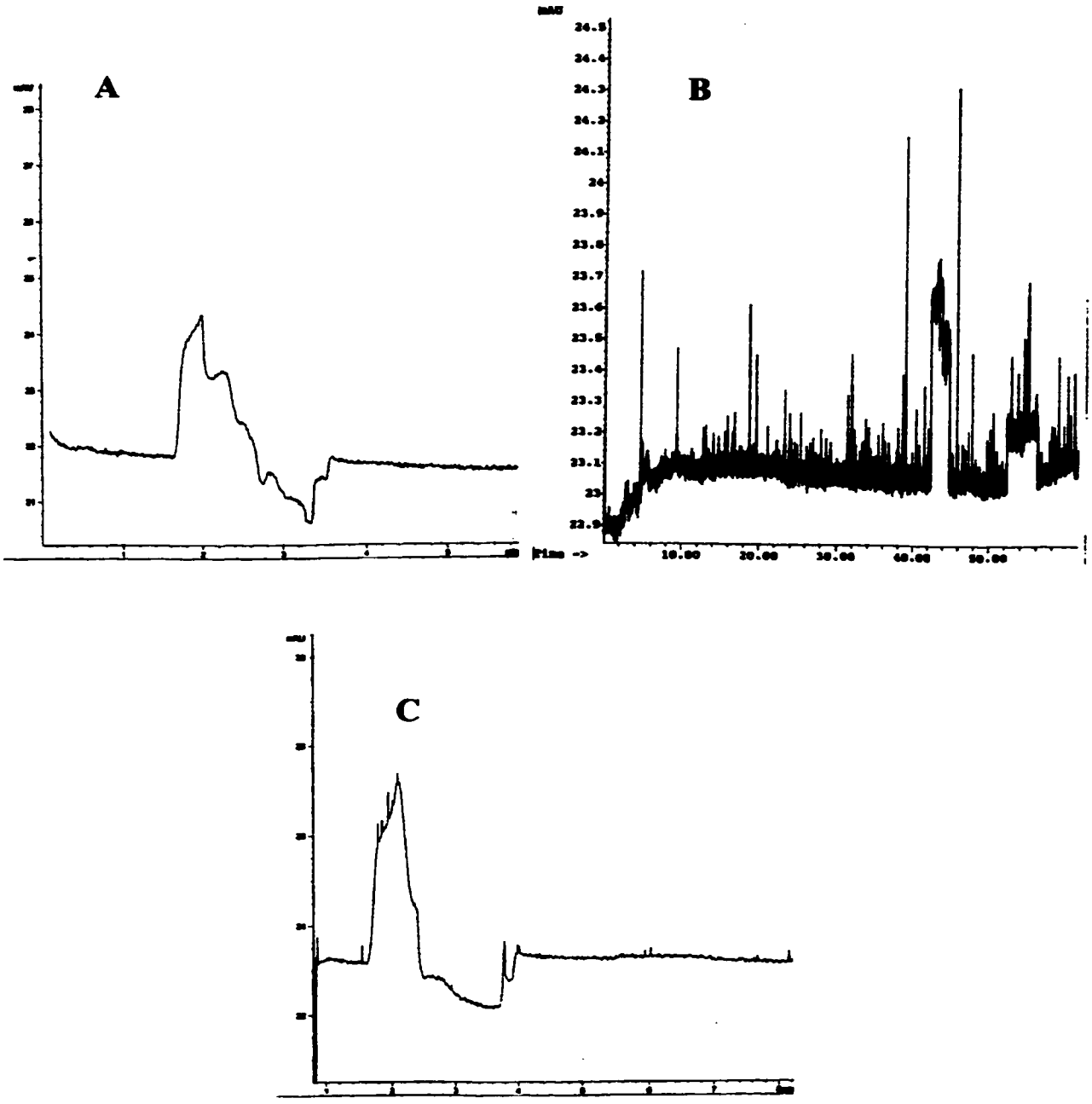


Figure 33 - Separation of erythromycin using 50 μm etched, C18, and cholesteryl-modified capillaries at pH 8.14. (A) etched (1:5): 20 kV, 2 μA, 20 sec, 211 nm. (B) C18 (1:5): 20 kV, 2 μA, 10 sec, 290 nm. (C) cholesteryl (1:5): 20 kV, 2 μA, 30 sec, 211 nm.

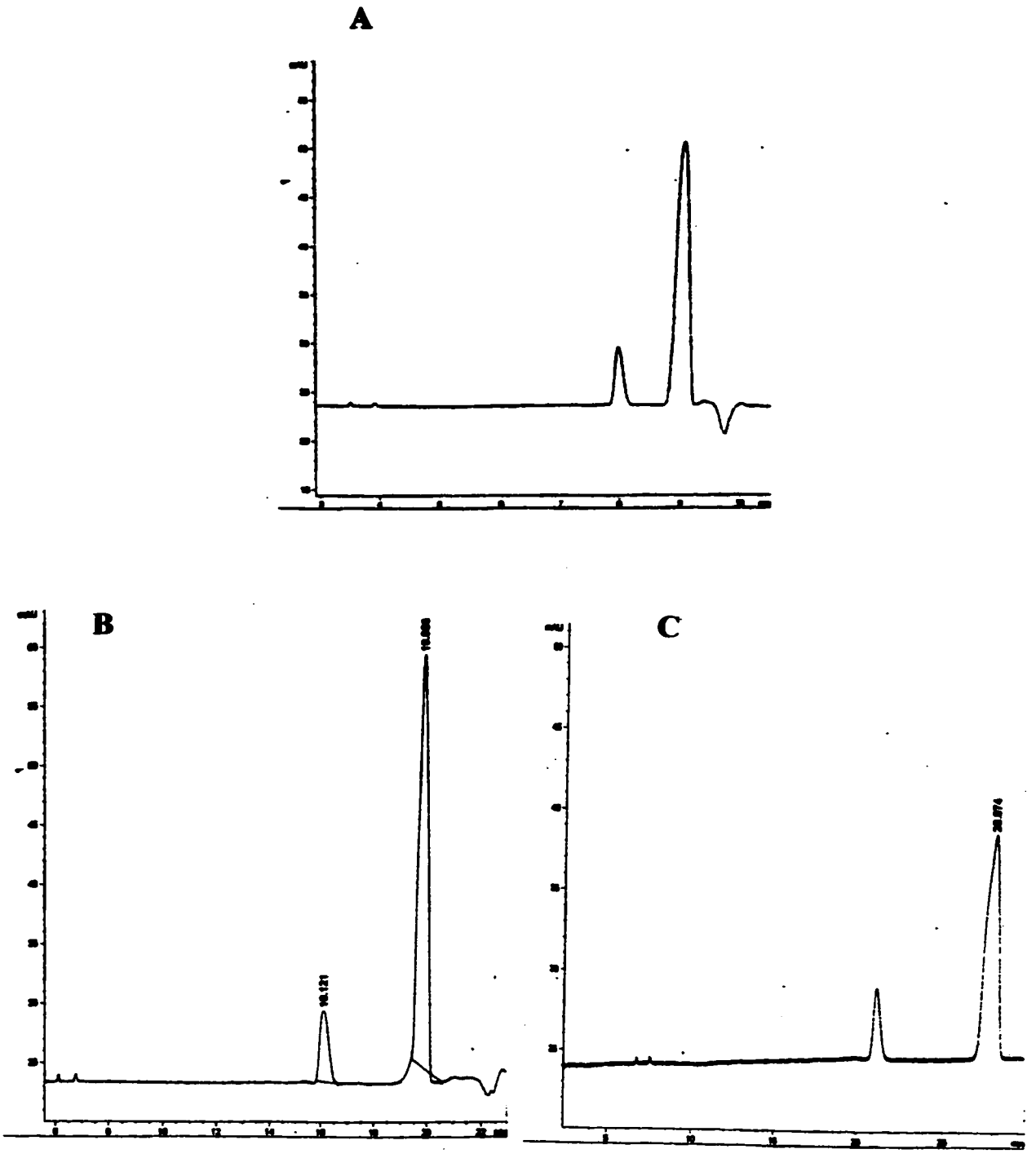


Figure 34 - Separation of ampicillin using 50 μm etched and fluorosurfactant-coated capillaries at pH 4.41 (1:10). (A) etched: 20 kV, 13 μA, 0.5 sec, 211 nm. (B) FSA: 20 kV, 11 μA, 0.6 sec, 211 nm. (C) FSK: 20 kV, 12 μA, 0.5 sec, 211 nm.

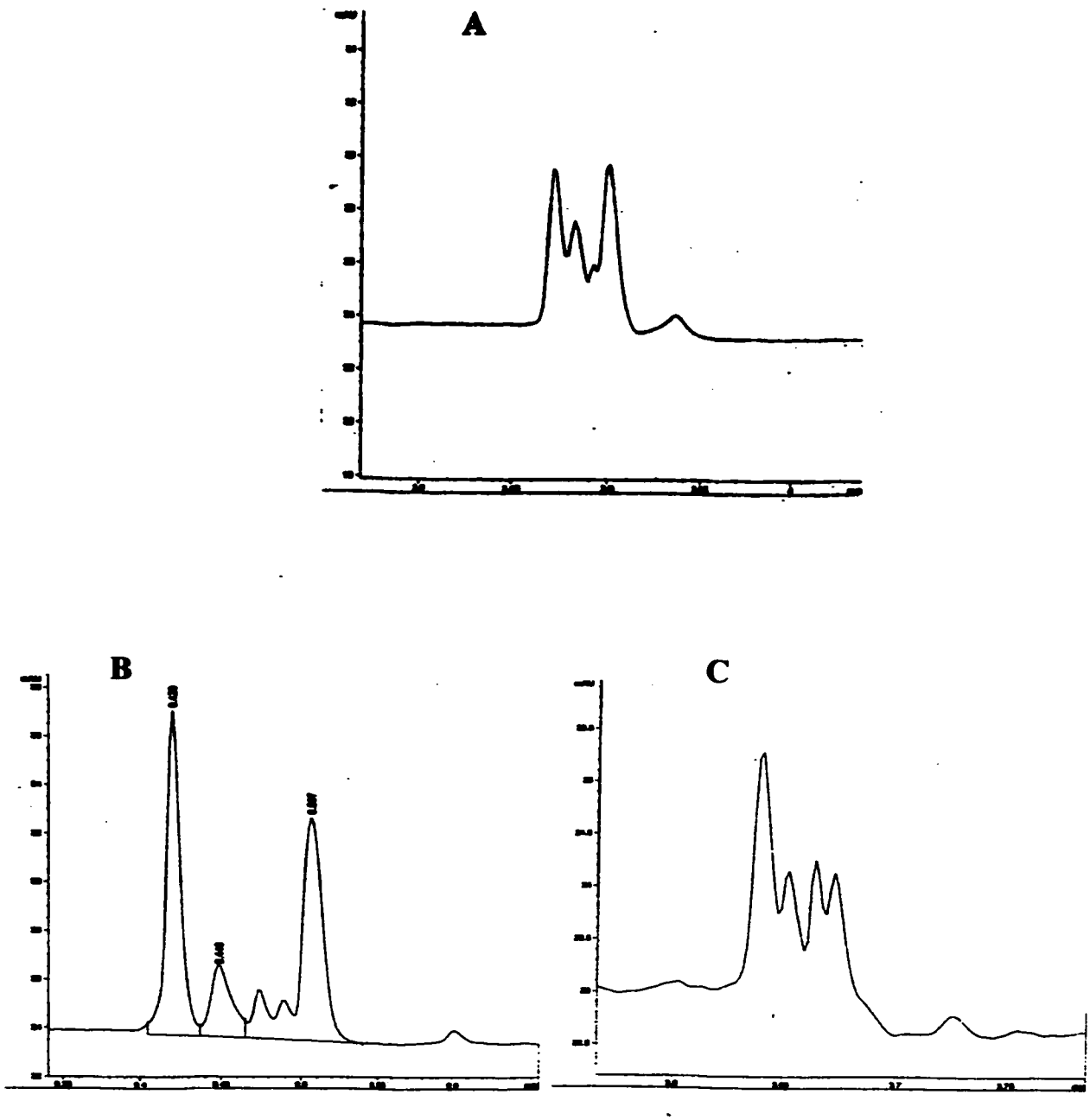


Figure 35 - Separation of gentamycin using 50 μm etched and fluorosurfactant-coated capillaries at pH 4.41 (1:10). (A) etched: 20 kV, 13 μA, 10 sec, 254 nm (B) FSA: 20 kV, 11 μA, 1 sec, 290 nm. (C) FSK: 20 kV, 13 μA, 1 sec, 290 nm.

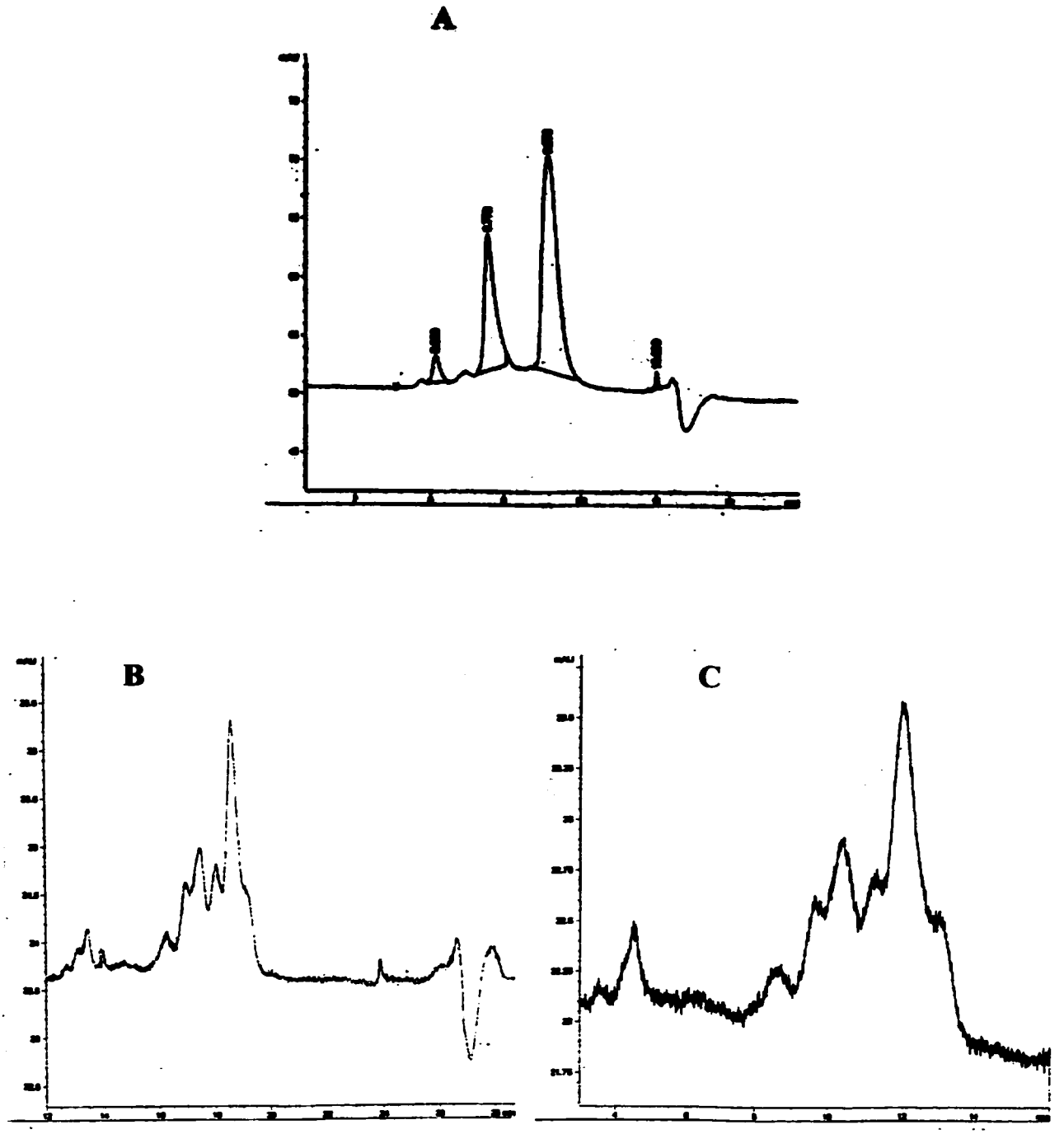


Figure 36 - Separation of nystatin using 50 μm fluorosurfactant-coated capillaries at pH 4.41 (1:10). (A) etched: 20 kV, 11 μA, 2 sec, 290 nm (B) FSA: 20 kV, 11 μA, 1 sec, 290 nm (C) FSK: 20 kV, 13 μA, 1 sec, 290 nm.

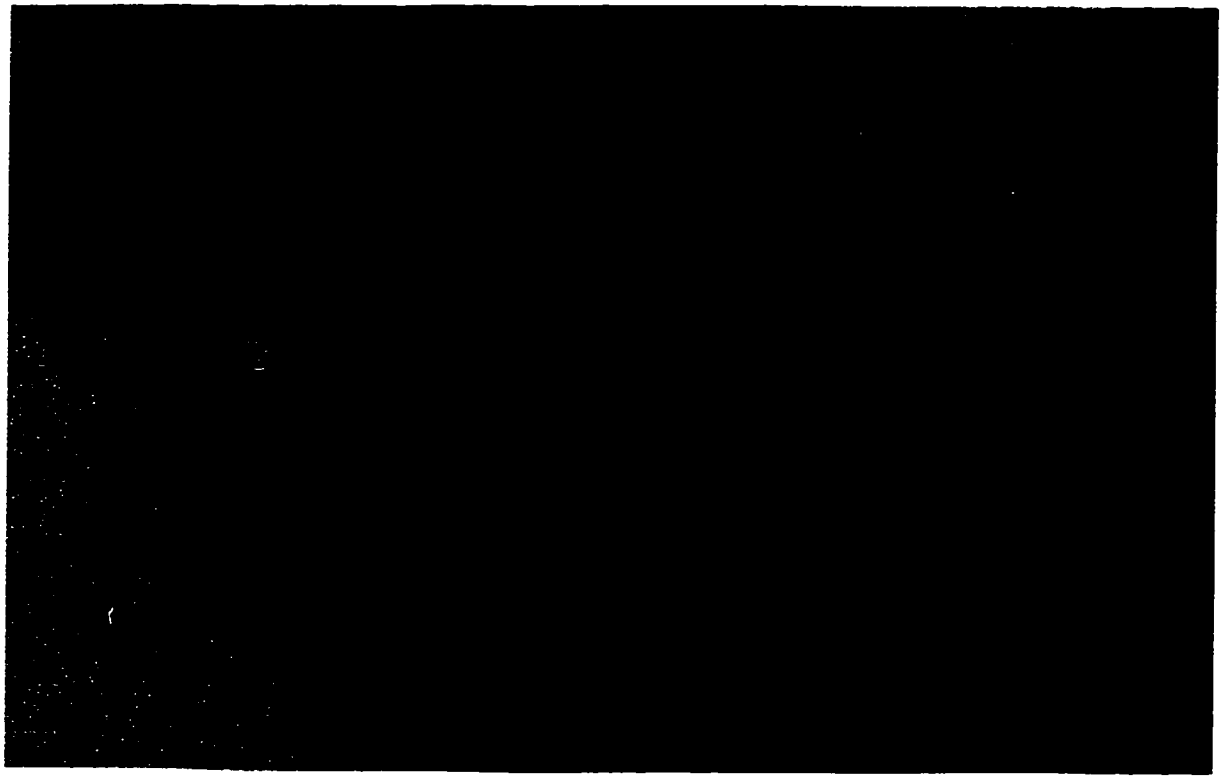


Figure 37 - An optical image of a 50 um capillary fragment with an AFM cantilever scanning the exposed inner-bore surface (Frommer, IBM).

Figure 38 - AFM image of a C18-modified capillary inner-wall surface. Scan rate = 1 Hz. (A) 5 x 5 μm, z = 500 nm (B) 3 x 3 μm, z = 500 nm (C) 1 x 1 μm, z = 50 nm where z is light-intensity scale (Frommer, IBM).

Figure 39 - AFM image of a cholesteryl-modified capillary inner-wall surface. Scan rate = 1 Hz. (A) 5 x 5 μm, z = 1 μm (B) 1 x 1 μm, z = 250 nm where z is light-intensity scale (Frommer, IBM).

Figure 40 - An optical image of capillary with AFM cantilever scanning the external surface (Frommer, IBM).

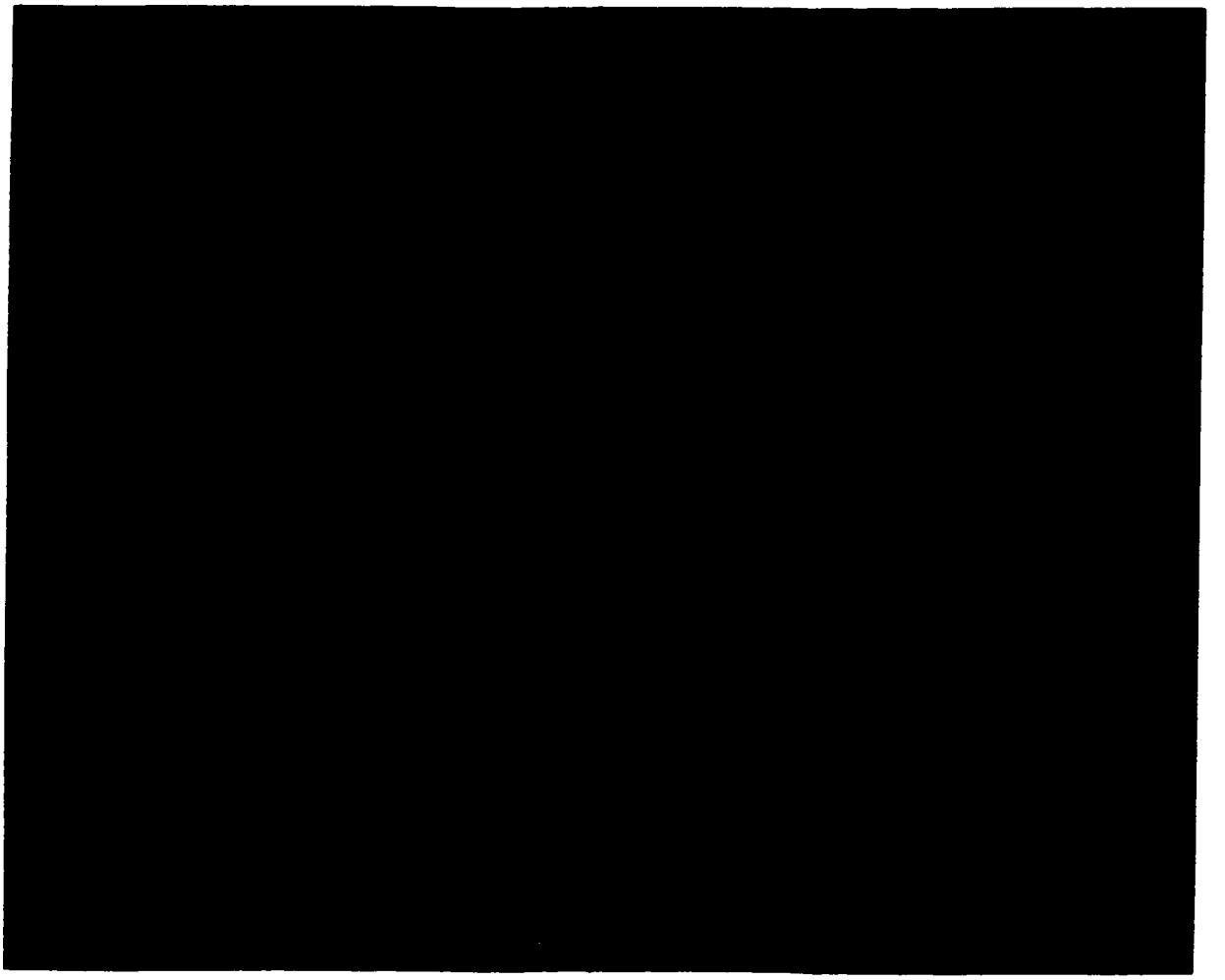


Figure 41 - An optical image of bare capillary when focus is on top of bore (Frommer, IBM).

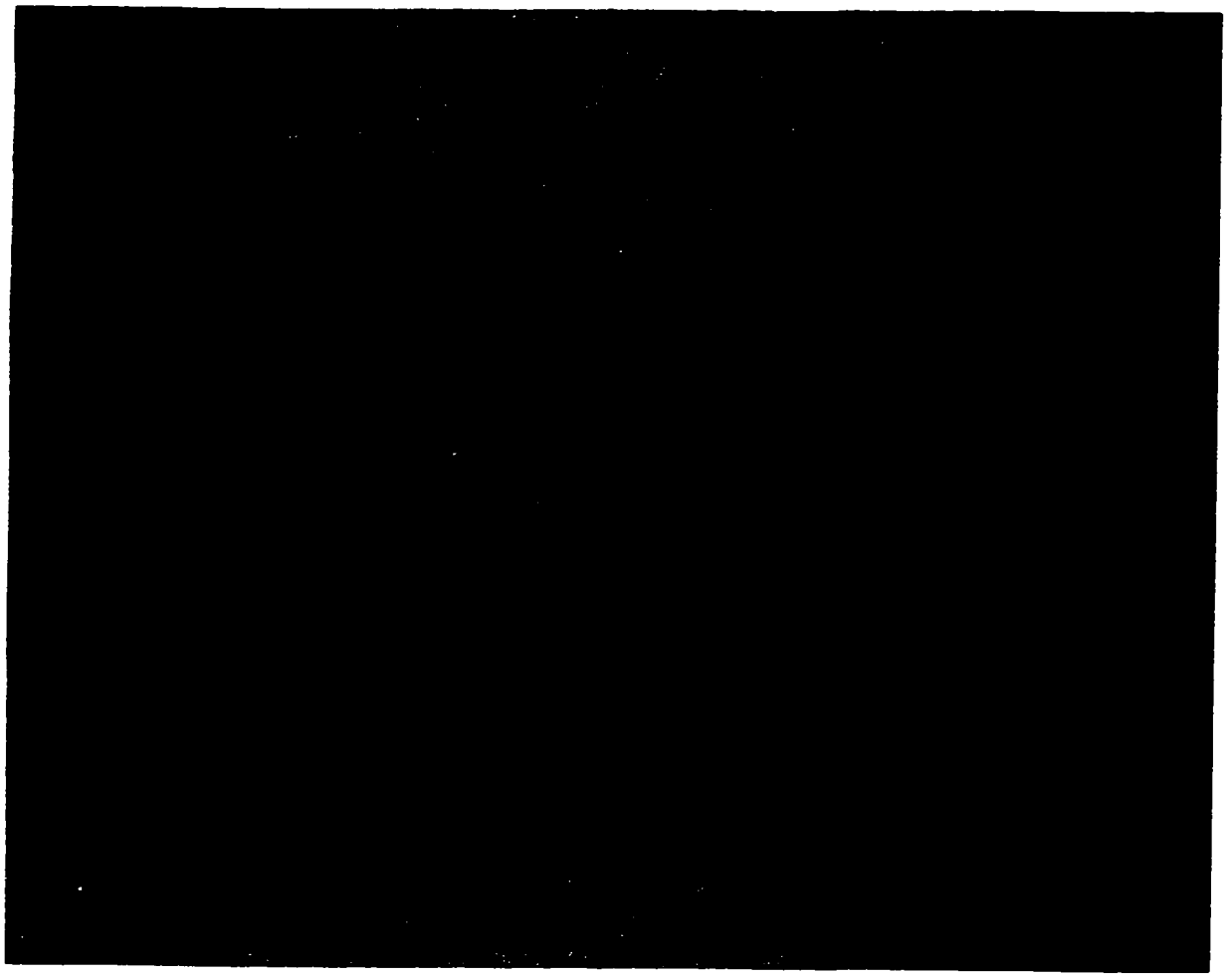


Figure 42 - An optical image of bare capillary when focus is under bore (Frommer, IBM).

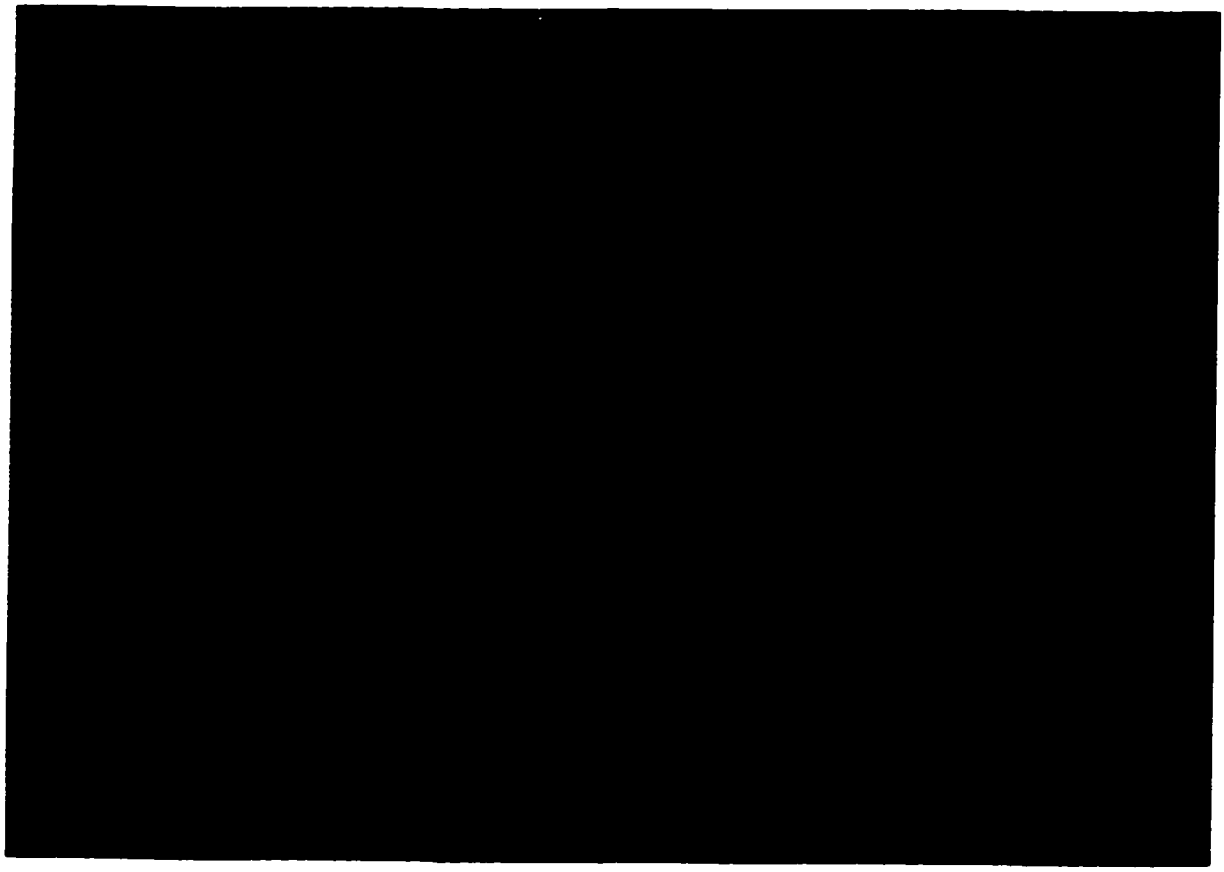


Figure 43 - An optical image of the externally etched capillary surface (Frommer, IBM).

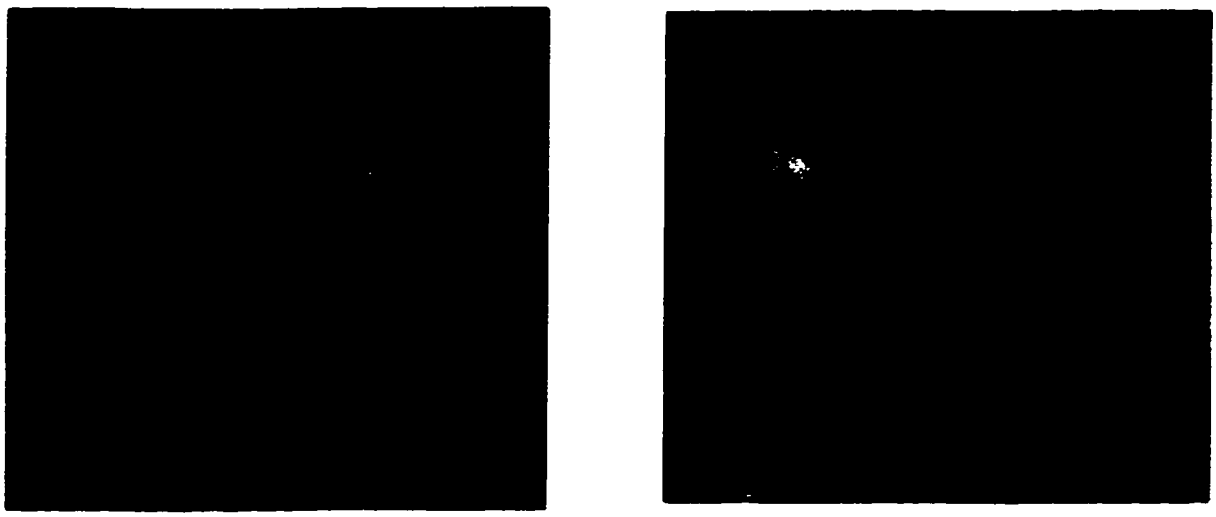


Figure 44 - AFM image of bare capillary outer-wall surface. Scan rate = 1 Hz. (A) 5 x 5 μm, z = 25 nm (B) 1 x 1 μm, z = 100 nm where z is light-intensity scale (Frommer, IBM).

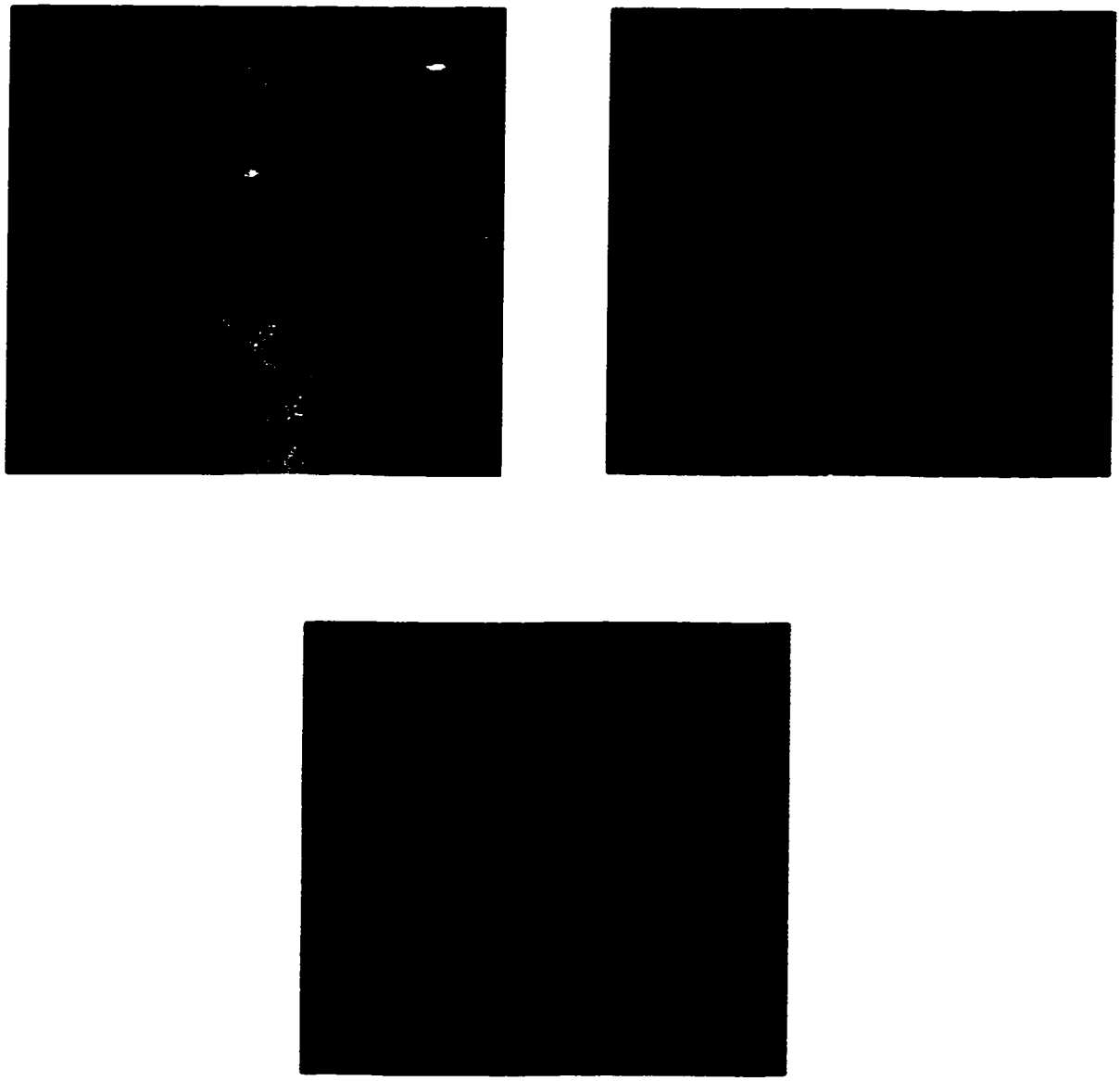


Figure 45 - AFM image of externally etched capillary surface. Scan rate = 1 Hz. (A) 25 x 25 μm, z = 1 μm, scan rate = 0.5Hz (B) 5 x 5 μm, z = 250 nm (C) 1 x 1 μm, z = 250nm where z is light-intensity scale (Frommer, IBM).

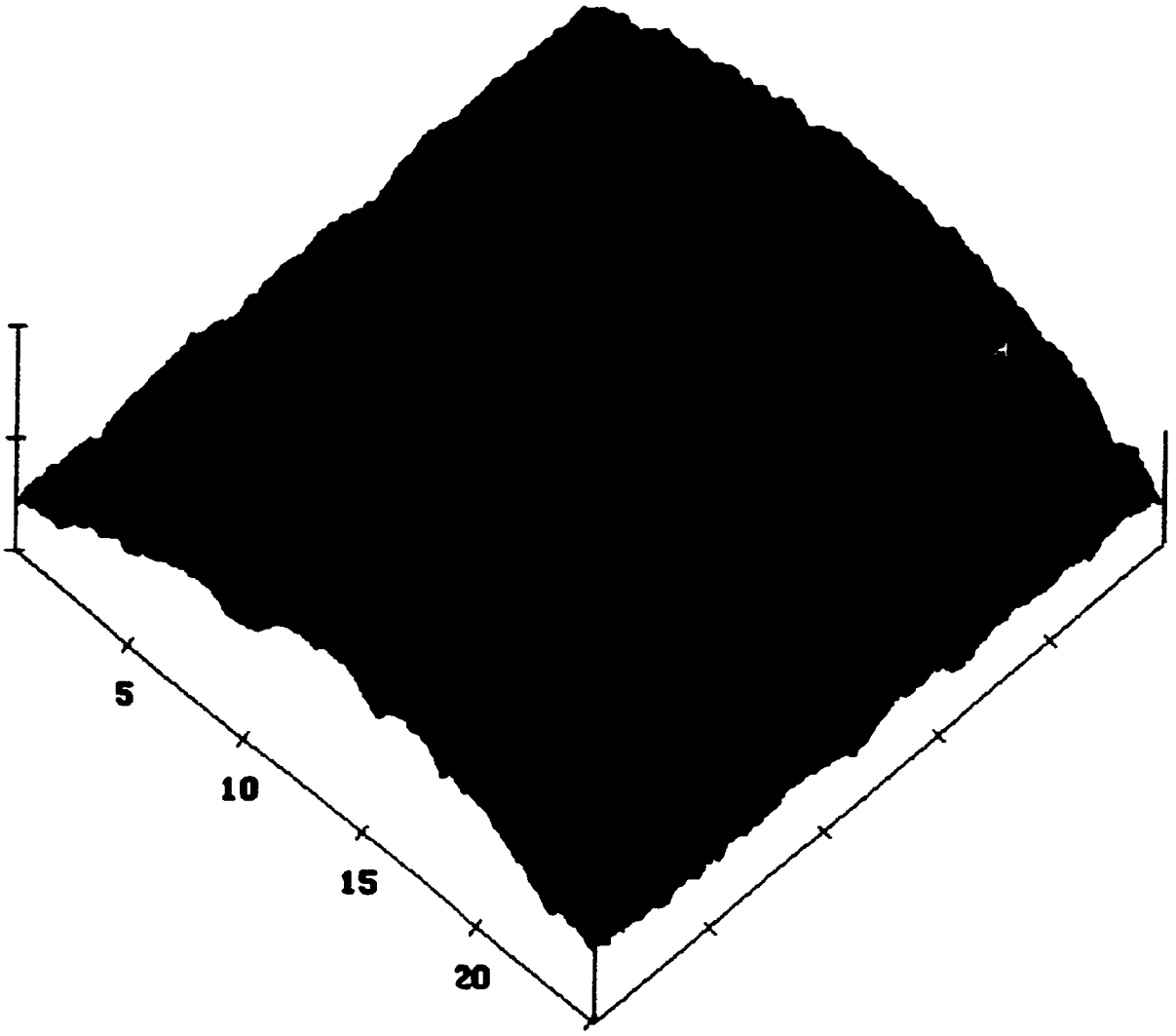


Figure 46 - AFM 3D-image of the externally etched capillary surface. Scan rate = 0.5Hz; size = 25 x 25 μm; z = 1 μm where z is the light-intensity scale (Frommer, IBM).

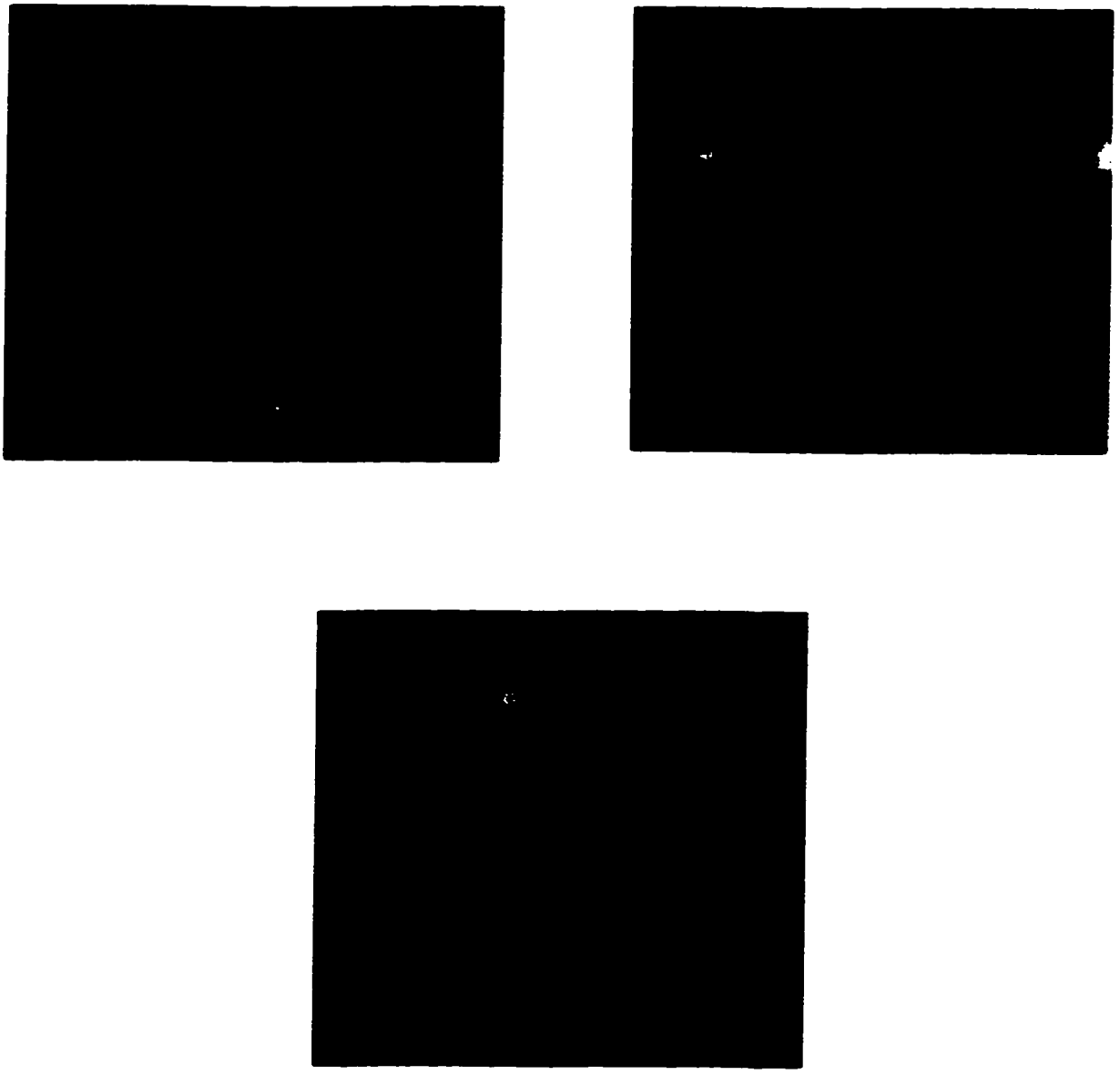


Figure 47 - AFM image of externally etched cholesteryl modified capillary surface. Scan rate = 1 Hz. (A) 10 x 10 μm, z = 25 nm (B) 3 x 3 μm, z = 25 nm (C) 50 x 50 nm, z = 25nm where z is light-intensity scale (Frommer, IBM).

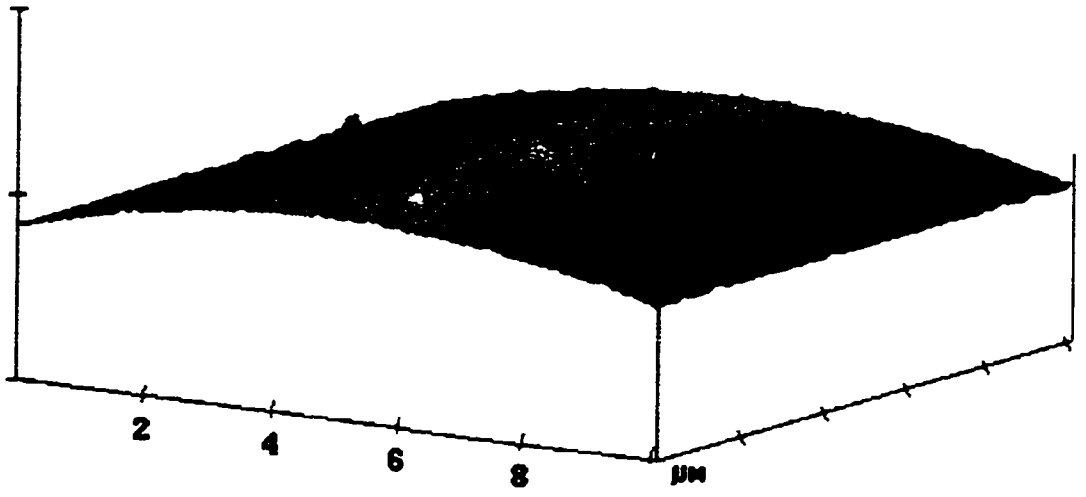


Figure 48 - AFM 3D-image of an externally modified capillary surface (cholesteryl-modified). Scan rate = 1 Hz; size = 10 x 10 μm; z = 250nm where z is the light-intensity scale (Frommer, IBM).

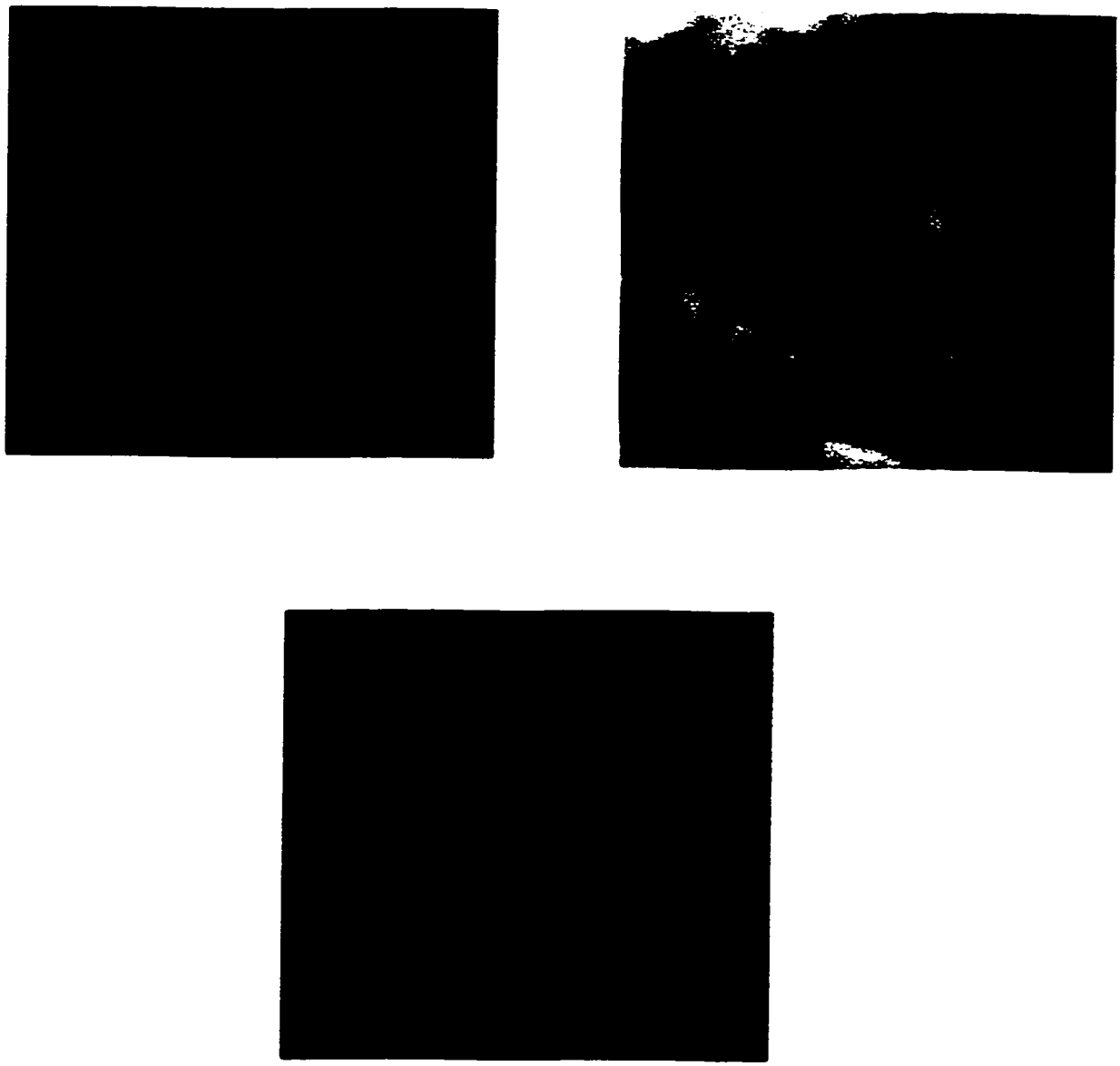


Figure 49 - AFM image of bare and externally modified capillary surfaces. Scan rate = 1 Hz. Size = 5 x 5 μm, z = 250 nm (A) bare (B) etched (C) cholesteryl-modified where z is light-intensity scale (Frommer, IBM).

Figure 50 - AFM image of bare and externally modified capillary surfaces. Scan rate = 1 Hz. Size = 1 x 1 μm, z = 100 nm (A) bare (B) etched (C) cholesteryl-modified where z is light-intensity scale (Frommer, IBM).